

Identification of Differentially Regulated Genes in Bovine Blastocysts Using an Annealing Control Primer System

KYU-CHAN HWANG,¹ XIANG-SHUN CUI,¹ SE-PILL PARK,² MI-RA SHIN,¹ SAE-YOUNG PARK,² EUN-YOUNG KIM,² AND NAM-HYUNG KIM^{1*}

¹Department of Animal Science, Chungbuk National University, Chungbuk, South Korea

²Maria Infertility Hospital Medical Institute/Maria Biotech, Seoul, South Korea

ABSTRACT The identification of embryo-specific genes would provide insights into early embryonic development. However, the current methods employed to identify the genes that are expressed at a specific developmental stage are labor intensive and suffer from high rates of false positives. Here we employed a new and accurate reverse transcription-polymerase chain reaction (RT-PCR) technology that involves annealing control primers (ACPs) to identify the genes that are specifically or prominently expressed in bovine early blastocysts and hatched blastocysts produced *in vitro*. Using these techniques, a total of nine expressed sequence tags (ESTs) of genes that were differentially expressed in hatched blastocysts, as compared to blastocyst embryos, were cloned and sequenced. The cloned genes or ESTs (C1–C9) all exhibited significant sequence similarity with known bovine genes (99–100%; FTL, RPS12, LAPTM4a, and RPL12) or ESTs (80–94%; AIBP, CULLIN-1, HDLP, COX5a, and RECS1) of other species. As revealed by real time RT-PCR, these genes were regulated upstream in the hatched blastocyst stage during early implantation. These results suggest that this new, PCR-based differential display RT-PCR technique is a very useful tool for the identification of stage-specific genes of preimplantation embryos. *Mol. Reprod. Dev.* 69: 43–51, 2004. © 2004 Wiley-Liss, Inc.

Key Words: bovine embryo; blastocyst; hatched blastocyst; ACP system

INTRODUCTION

In mammals, preimplantation development is characterized by various morphological and physiological transitions that occur following fertilization. The zygote undergoes several cleavage divisions, following which it compacts and cavitates to form a blastocyst and then hatches from zona pellucida. Early in implantation, embryos utilize transcripts and proteins synthesized during oogenesis until they reach the stage at which the embryonic genome becomes activated (Schultz et al., 1999). This nonpermissive period of transcription differs

significantly between species. In bovine embryos, embryonic gene activation has occurred definitively by the 8- to 16-cell stage, along with major changes in the ultrastructure of blastomere nucleoli and in patterns of protein synthesis (Camous et al., 1986; King et al., 1988; Frei et al., 1989; Kopecny, 1989, 1996). Further development is dependent on the successful regulation of both temporal and spatial gene expression following activation of the embryonic genome.

Thousands of genes have to be expressed in a stage-specific manner to ensure normal embryo development. However, of this myriad of developmentally regulated genes, to date only few have been characterized with respect to physiological function (Niemann and Wrenzycki, 2001). Therefore, to further understand the molecular basis of preimplantation development, it would be of interest to identify and perform detailed characterization of differentially expressed genes (DEGs). In many cases, our basic understanding of gene expression during early bovine embryogenesis has been gained from data extrapolated from the mouse. Thus, the identification of novel genes and analysis of their function during bovine preimplantation embryogenesis is necessary. To this end, several reverse transcription-polymerase chain reaction (RT-PCR) technologies have been applied to the identification of embryonic genes or expressed sequences, however in general these techniques are labor-intensive and have a high degree of false positives. For example, differential display RT-PCR was used to compare patterns of RNA expression from preattachment bovine embryos (Natale and Watson,

K.C. Hwang, X.S. Cui, and S.P. Park contributed equally to this work. Grant sponsor: Korea Health 21 R&D Project, Ministry of Health & Welfare; Grant number: 01-PJ10-PG8-01EC01-0010; Grant sponsor: Research Center for Bioresource and Health and KOSEF Republic of Korea.

*Correspondence to: Nam-Hyung Kim, PhD, Department of Animal Sciences, Chungbuk National University, Gaesin-dong, Cheongju, Chungbuk, 361-763, South Korea. E-mail: nhkim@chungbuk.ac.kr

Received 21 February 2004; Accepted 22 March 2004

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mrd.20156

2002). Furthermore, analysis of cDNA libraries derived from oocytes is only as robust as the quality of the libraries and subtraction methods are biased towards abundant transcripts. Recently, suppression subtractive hybridization has been used to identify genes in preimplantation mammalian embryos (Mohan et al., 2002).

The success of PCR amplification relies solely on the specificity with which a primer anneals to its target sequences. Therefore, it is important to optimize this molecular interaction (McPherson and Moller, 2000). The annealing temperature is critical for determining whether a primer binds only to its perfect complement or to sequences with one or more mismatches. By adjusting the annealing temperature, one can alter the specificity of pairing between template and primer. Numerous approaches, such as longer primers with universal, homopolymer, or loop sequence tails at their 5'-ends, have been introduced to increase primer annealing specificity (Saiki et al., 1989; Brownie et al., 1997; Ailenberg and Silverman, 2000). However, these approaches do not abrogate nonspecific hybridization resulting from the involvement of the tail or loop sequence in the priming reaction. We describe here a most accurate and extensive PCR technology, controlled by an annealing control primer (ACP), that specifically targets sequence hybridization to the template via a polydeoxyinosine [poly (dI)] linker (Hwang et al., 2003). The basis of ACP technology is the unique tripartite structure of a specific oligonucleotide primer (ACP), which has 3'- and 5'-end distinct portions separated by a regulator, and the interaction of each portion of this primer during a two-stage PCR (Fig. 1).

In the present study, we used a new differential display method, termed an ACP system, to analyze differentially expressed genes (DEGs) in bovine blastocysts and hatched blastocysts produced *in vitro*. We confirmed the expression patterns of 9 DEGs by RT-PCR assay, and quantified their expression throughout the

preimplantation stages by fluorescence monitored real time RT-PCR. These results suggest that this new PCR-based DDRT-PCR technique is a very useful tool for the identification of stage-specific DEGs of preimplantation embryos.

MATERIALS AND METHODS

In Vitro Production of Bovine Blastocysts

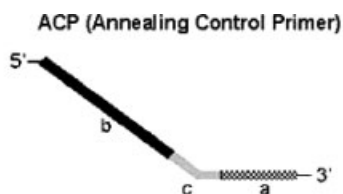
Cumulus-oocyte complexes (COCs) were aspirated from visible follicles (2–6 mm in diameter) from slaughterhouse ovaries. The COCs were then washed with HEPES-buffered Tyrode's medium and cultured in maturation medium composed of TCM199 + 10% fetal bovine serum (FBS) supplemented with 0.2 mM sodium-pyruvate, 1 µg/ml follicle-stimulating hormone, 1 µg/ml estradiol-17β, and 25 µg/ml gentamycin sulfate at 39°C, 5% CO₂ incubator. After incubation for 22–24 hr in IVM medium, the COCs were inseminated using highly motile sperm recovered from frozen-thawed bull semen separated on a discontinuous percoll column. Fertilization was assessed as cleavage rate (≥2-cell) after 44 ± 2 hr coincubation with the sperm. For *in vitro* culture, cleaved embryos were cultured in CR1 (Rosenkrans et al., 1993) medium supplemented with 3 mg/ml fatty acid-free BSA and then transferred into 10% FBS added CR1 medium at day 4 after IVF.

mRNA Extraction

The mRNA samples were prepared from pools of blastocyst stage embryos (Bl; n = 10) and hatched blastocyst embryos (Hbl; n = 10) for RT-PCR, Bl (n = 120) and Hbl (n = 60) for ACP-based differential display, and *in vitro*-produced 1-cell (n = 160), 2-cell (n = 80), 4-cell (n = 40), 8-cell (n = 20), 16-cell (n = 10), morula (n = 5), blastocyst (n = 5), and hatched blastocyst (n = 5) stages for real time RT-PCR using an oligo(dT)₂₅ nucleotide attached to magnetic beads (Dynabeads mRNA purification kit; Dynal, Oslo, Norway) following the manufacturer's instructions. Briefly, embryos were resuspended in 100 µl lysis/binding buffer (100 mM Tris-HCl with pH 7.5, 500 mM LiCl, 10 mM EDTA with pH 8.0, 1% LiDS, 5 mM DTT), and vortexed at room temperature for 5 min to facilitate the lysis of the embryo and release of RNA. Fifty microliters of oligo(dT)₂₅ magnetic bead suspension was added to the samples, and they were incubated at room temperature for 5 min. The hybridized mRNA and oligo(dT) magnetic beads were washed twice with washing buffer A (10 mM Tris-HCl with pH 7.5, 0.15 M LiCl, 1 mM EDTA, 1% LiDS) and once with washing buffer B (10 mM Tris-HCl with pH 7.5, 0.15 M LiCl, 1 mM EDTA), respectively. Finally, mRNA samples were eluted in 15 µl double-distilled DEPC-treated water.

RT-PCR

For the first strand cDNA synthesis, oligo(dT)_{12–18} primer was added to the mRNA solution isolated from blastocyst stage and Hbl using oligo (dT) attached magnetic beads (Dynabeads mRNA purification kit). The RT mix contained 1 × RT buffer, 10 mM dithio-



Designation	Function	Sequence length (nts)
a. Core sequence	Annealing at first stage of PCR	10
b. Universal sequence	Annealing at first stage of PCR	22
c. Regulator	Regulating the functions of a and b	5

Fig. 1. Annealing control primer (ACP) structure. The structure of ACP is based on tripartite sequence regions: core sequence (3'-end targeting portion; 10 nts) having a hybridizing sequence substantially complementary to a site on a template nucleic acid to hybridize; universal sequence (5'-end portion; 22 nts); and regulator sequence (linker portion separated 3-end portion and 5'-end portions; 5 nts). For generating a specific PCR product, the regulator sequence specifically controls annealing of the core sequence at the first stage of PCR and annealing of the universal sequence at the second stage of PCR by temperature, 50 or 65°C, respectively.

threitol (DTT), 0.5 mM of each dNTP, 10 U RNasin ribonuclease inhibitor, and 200 U of SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). The reverse transcription reaction was carried out at 42°C for 90 min followed by heating at 94°C for 2 min to inactivate the reaction. After the reverse transcription (RT) reaction, the final volume was increased to 50 µl with ultra-purified water, then stored all cDNA samples at -20°C until use in PCR. PCR analysis were performed in a final volume of 50 µl containing 5 µl of the cDNA sample, 2 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.2 mM of each dNTP, 0.4 µM of each primer, and 2 U rTaq polymerase (TaKaRa, Shiga, Japan). The amplification of expressed sequence tags (ESTs) cloned from ACP differential display was performed with specific primers, and these are indicated in Table 2. For each RT-PCR analysis, internal histone H2a and negative (blank) controls were included. After a first denaturation step of 3 min at 94°C, 30–38 amplification cycles were performed. Each cycle involved denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final extension step of 5 min at 72°C was performed to complete the PCR reaction. Then, 25% of the PCR product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized by exposure to ultraviolet light. The images were obtained using GELMANAGER apparatus (Prime-Tech, Seoul, Korea).

ACP-Based Differential Display

First-strand cDNA synthesis. Messenger RNA (mRNA) extracted from blastocyst stage and hatched blastocyst stage embryos using oligo(dT) attached magnetic beads (Dynabeads mRNA purification kit) were used for the synthesis of first-strand cDNAs by reverse transcriptase, as described by Hwang et al. (2003). Reverse transcription was performed for 1.5 hr at 42°C in a final reaction volume of 20 µl containing the purified mRNA, 4 µl of 5 × reaction buffer (Promega, Madison, WI), 5 µl of dNTP (each 2 mM), 2 µl of 10 µM cDNA synthesis primer dT-ACP1 (Table 1), 0.5 µl of RNasin Plus RNase Inhibitor (40 U/µl; Promega), and 1 µl of Superscript II reverse transcriptase (200 U/µl; Invitrogen, Carlsbad, CA). First-strand cDNAs were diluted by the addition of 120 µl of ultra-purified water.

ACP-based PCR. Second-strand cDNA synthesis and subsequent PCR amplification were conducted in a single tube. Second-strand cDNA synthesis was conducted at 50°C (low stringency) during one cycle of first-stage PCR in a final reaction volume of 49.5 µl containing 1 µl of the diluted first-strand cDNA, 5 µl of 10 × PCR reaction buffer (Roche Applied Science, Mannheim, Germany), 5 µl of dNTP (each 2 mM), 1 µl of 10 µM dT-ACP2 (Table 1), and 1 µl of 10 µM arbitrary primer (Table 1) preheated to 94°C. The tube containing the reaction mixture was held at 94°C while 0.5 µl of Taq DNA Polymerase (5 U/µl; Roche Applied Science) was added to the reaction mixture. The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, 40 amplification cycles were performed. Each cycle involved denaturation at 94°C for 40 sec, annealing at 65°C for 40 sec, and extension at 72°C for 40 sec. A final extension step of 5 min at 72°C was performed to complete the PCR. The amplified PCR products were separated on 2% agarose gels and stained with ethidium bromide.

Cloning and transformation. The differentially expressed bands were extracted and cloned into a TOPO TA cloning vector (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. In order to verify the identity of insert DNA, isolated plasmids were sequenced automatically (Applied Biosystems, California). Complete sequences were analyzed by searching for similarities using BLASTX search program at the National Center for Biotechnology Information (NCBI) GenBank.

Real Time RT-PCR Quantification

To validate the results of ACP differential display and to determine the relative abundance of target sequences, real time quantitative PCR was performed using mRNA isolated from pools of in vitro-produced 1-cell, 2-cell, 4-cell, 8-cell, 16-cell, morula, blastocyst, and hatched blastocyst stages. Due to the relatively small number of embryos, RNA isolation was performed using oligo(dT) attached magnetic beads (Dynabeads mRNA purification kit) following the manufacturer's instructions. Briefly, embryos were resuspended in 300 µl lysis/binding buffer (100 mM Tris-HCl with pH 7.5, 500 mM

TABLE 1. Primer Sequence Used in cDNA Synthesis and ACP-Based PCR

Use	Primer name	Sequence
cDNA synthesis primer	dT-ACP1	5'-CTGTGAATGCTGCGACTACGAT <u>TTTT</u> (T) ₁₈ -3'
Reverse primer	dT-ACP2	5'-CTGTGAATGCTGCGACTACGAT <u>TTTT</u> (T) ₁₅ -3'
Arbitrary primer (forward primer)	ACP5	5'-GTCTACCAGGCATTGCTTCAT <u>TTTT</u> AGTGCCTCG-3'
	ACP16	5'-GTCTACCAGGCATTGCTTCAT <u>TTTT</u> GTGACGGTG-3'
	ACP24	5'-GTCTACCAGGCATTGCTTCAT <u>TTTT</u> CCACCGTGTG-3'
	ACP28	5'-GTCTACCAGGCATTGCTTCAT <u>TTTT</u> TGGGAACCGG-3'
	ACP34	5'-GTCTACCAGGCATTGCTTCAT <u>TTTT</u> GCATCCAGCA-3'
	ACP43	5'-GTCTACCAGGCATTGCTTCAT <u>TTTT</u> CTAAACCAA-3'
	ACP89	5'-GTCTACCAGGCATTGCTTCAT <u>TTTT</u> TCCCAATGGG-3'
	ACP113	5'-GTCTACCAGGCATTGCTTCAT <u>TTTT</u> CAGCTCGTGG-3'

ACP, annealing control primer

The polydeoxyinosine [poly(dI)] linkers are underlined. I represents deoxyinosine.

LiCl, 10 mM EDTA with pH 8.0, 1% LiDS, 5 mM DTT), and vortexed at room temperature for 5 min to facilitate the lysis of the embryo and release of RNA. 50 µl of oligo(dT) magnetic bead suspension was added to the samples, and incubated at room temperature for 5 min. The hybridized mRNA and oligo(dT) magnetic beads were washed two times with washing buffer A (10 mM Tris-HCl with pH 7.5, 0.15 M LiCl, 1 mM EDTA, 1% LiDS) and washing buffer B (10 mM Tris-HCl with pH 7.5, 0.15 M LiCl, 1 mM EDTA), respectively. Finally, mRNA samples were eluted in 30 µl double-distilled DEPC-treated water and the reverse transcription (RT) reaction was carried out in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 6 mM MgCl₂, 2 mM DTT, 1 mM of each dNTPs, 20 U of RNase inhibitor, and 200 U of Super-script II (Invitrogen, Carlsbad, CA). The reaction mixture was incubated at 42°C for 90 min and then, 94°C for 2 min.

Prior to real time PCR amplification, sequence-specific primers were designed from DEGs cloned from ACP differential display using Primer3 Software v0.2c (<http://www.broad.mit.edu/cgi-bin/primer/primer3.cgi>). For optimal quantification, parameters were set to design primer sequences with melting temperature (T_m) of 55–65°C, no 3'-end complementarity (to avoid primer-dimer formation) and product size of 150–300 bp. The sequence and the product size of all specific primers are listed in Table 2. The PCRs were conducted in DNA Engine OPTICON2 (MJ Research, San Francisco, CA) and were detected with SYBR Green as a double-stranded DNA-specific fluorescent dye, which is included in the SYBR Green qPCR premix (FINN-ZYMES). Prior to the quantification, optimization procedures were performed by running PCRs, with or without the purified template, to identify the melting temperatures of the primer dimers and the specific

product. To measure the level of mRNA in the samples, the fluorescence values were taken at a temperature associated with the beginning of the peak for the specific product (Table 2). Standard curves were generated for both target and internal control genes using serial dilutions of plasmid DNA (10²–10⁷ molecules). PCRs were performed in 20 µl reaction buffer containing 10 µl 2× SYBR Green premix, 1 µl of forward and reverse primers (5 pmole/µl) and 1 µl embryonic cDNA (0.1 blastocyst/µl equivalent). Each PCR run was performed in triplicate to control the reproducibility of quantitative results. The amplification program was as following: preincubation for HotStart polymerase activation at 95°C at 15 min, followed by 45 amplification cycles of denaturation at 95°C for 1 min (2°C/sec), annealing at 55°C for 1 min (2°C/sec), elongation at 72°C for 1 min (2°C/sec), and acquisition of fluorescence at 72°C or 80°C for 1 sec. After the end of the last cycle, the melting curve was generated by starting the fluorescence acquisition at 65°C and taking measurements every 0.2°C until 95°C was reached. The results were reported as the relative expression or n-fold difference to the calibrator cDNA (i.e., the blastocyst stage) after normalization of the transcript amount to the endogenous control by 2-ddCt method (Livak and Schmittgen, 2001). The sizes of PCR products were confirmed by gel electrophoresis on a standard 1.5% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light.

RESULTS

Messenger RNA Expression Pattern

In order to identify and isolate DEGs in preimplantation stage embryos, we compared the mRNA expression profiles in in vitro-produced bovine embryos. mRNA isolated from each of the developmental stages (blas-

TABLE 2. Sequence-Specific Primers Used for Quantification of Differentially Expressed Transcripts

Clone name	Primer name	Sequence	Product length (bp)	Genbank accession number	Temperature of fluorescence acquisition (°C)
C1	Forward	5'-AGGCTGGGTTGGGCGAGTAT-3'	165	AY528246	84
	Reverse	5'-TGAGAGAGGGCTCCAGGGTG-3'			
C2	Forward	5'-GGGTGCATTCAAGATTCGGC-3'	248	AY528251	85
	Reverse	5'-CACAAAGGGCCTCCACCAAC-3'			
C3	Forward	5'-TGCCTGAAGAAATTCTGCCT-3'	250	AY528247	84
	Reverse	5'-ATGGCACTTTGCTCTTGCTT-3'			
C4	Forward	5'-CCACCATGCCCGCTAAGTTC-3'	158	AY528249	80
	Reverse	5'-ACCAGTTGCCTTGCGCATGT-3'			
C5	Forward	5'-TCATCTGTGGCCCTGGGAAT-3'	281	AY528250	81
	Reverse	5'-GACACTCAGGATGCTGCGGA-3'			
C6	Forward	5'-GGGCTGGTGTCCCTTCT-3'	225	AY528252	82
	Reverse	5'-AGGCACAGATCCCGTTCAGC-3'			
C7	Forward	5'-AAGGAAACATGGCCCTGGCA-3'	150	AY528253	78
	Reverse	5'-AATGGGGATGAAAAGAAACATTCA-3'			
C8	Forward	5'-TGATGCTCGCTGGGTGACAT-3'	288	AY528254	81
	Reverse	5'-TCAAGGCCAGTTCCTCTGG-3'			
C9	Forward	5'-CTTCTGCTTCCAGCAAGG-3'	263	AY528248	81
	Reverse	5'-CTGTGTAGATCTGCAGGGCA-3'			
H2a	Forward	5'-GTCTTGGAGTACCTGACCGC-3'	201	AW461431	81
	Reverse	5'-AGTCTTCTTCGGGAGCAACA-3'			

to cyst or hatched blastocyst) was subjected to the ACP system. For this, mRNA isolated from a pool of 120 blastocyst-stage embryos (Bl) or 60 hatched blastocyst-stage embryos (Hbl), using magnetic oligo(dT), was used as a template for first-strand cDNA synthesis using dT-ACP1 as a primer (Table 1). Using first-strand cDNA as templates, second-strand cDNAs were synthesized during one cycle of first-stage PCR using an arbitrary ACP primer (Table 1) and an initial annealing temperature of 50–53°C. In our approach, an arbitrary ACP primer and dT-ACP2 primer coexist in the same reaction tube, but the 3'-end core sequence of dT-ACP2 cannot anneal to the first-strand cDNA at the first annealing temperature required. However, this annealing temperature does permit the 3'-end core sequence (10-mer) of the arbitrary ACP to anneal to a specific template site. By using a combination of dT-ACP2 (reverse primer) and 120 arbitrary ACPs (forward primer), second-strand cDNAs are then amplified during second-stage PCR at a second annealing temperature (65°C), which constitute high-stringency conditions, using the sequences at the 3'- and 5'-ends of the second-strand cDNAs as templates for the amplification priming sequences. On the basis of differential expression levels of mRNA fragments observed on agarose gels, 9 bands were detected from Hbl as compared to Bl (blastocyst stage embryos) using eight arbitrary ACPs (Fig. 2A,B). They were excised from the gels, cloned and sequenced for analysis. The cloned DEGs were denoted C1 to C9 (Fig. 2A,B). The expression patterns of the DEGs were confirmed by RT-PCR (Fig. 3). The RT-PCR assays revealed that in agreement with the ACP differential display, transcripts C1–C9 had similar expression patterns when DEGs from Hbl were compared to Bl (blastocyst embryos). However, for DEG C1, C3, C4, C5, C8, and C9 of Bl, low-intensity bands were observed, relative to the ACP differential display (Fig. 3).

The functional role, sequence similarity, and characterization of differentially expressed transcripts are summarized in Table 3. BLASTN and BLASTX searches for sequence similarity in NCBI GenBank revealed that DEGs (C1–C9) showed significant similarity with known genes or ESTs (Table 3). Of these, AIBP (C5), CULLIN-1 (C6), HDLP (C7), COX5a (C8), and RECS1 (C9) shared similarity (80–94%) with sequences from other species (human, mouse, and rat or zebrafish, pig, and horse partially) whereas FTL (C1), RPS12 (C2), LAPTM4a (C3), and RPL12 (C4), showed significant similarity (99–100%) with the coding regions of known bovine genes (Table 3). All genes or ESTs identified and characterized in this study have already been submitted to Genbank and assigned accession numbers (Table 2).

Confirmation of ACP Differential Display by Real Time RT-PCR

To confirm the efficacy of the ACP system and to further investigate the stage-specific expression patterns of the identified genes or ESTs, fluorescence monitored quantitative real time RT-PCR analysis was

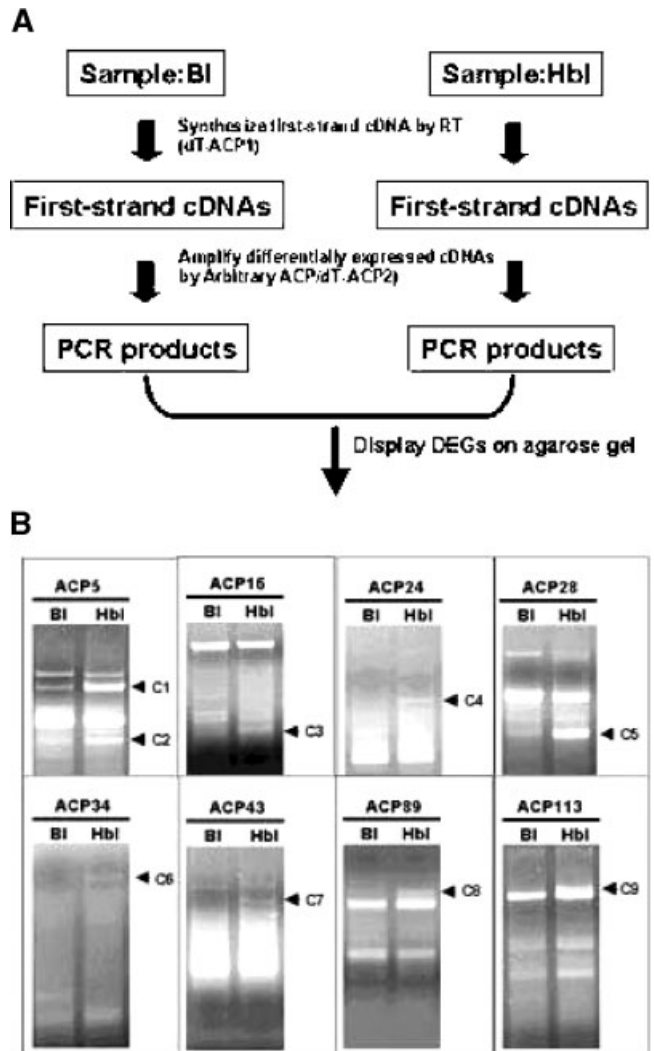


Fig. 2. Schematic illustration of methods using the annealing control primer (ACP) system and results of ACP-based PCR for identification of differentially expressed genes (DEGs) from two developmental stages. **A:** Messenger RNA (mRNA) from Bl and Hbl are used for the synthesis of first-strand cDNA using dT-ACP1. By using a combination of dT-ACP2 (reverse primer) and 120 arbitrary ACPs (forward primer), second-strand cDNAs are then amplified during second-stage PCR, and separated for differentially expressed genes (DEGs) on agarose gels. **B:** Gel are photographed to show differential banding patterns obtained from bovine blastocyst embryos (Bl) and hatched blastocyst embryos (Hbl) produced *in vitro* using a set of an arbitrary ACP (5'-primer) and dT-ACP2 (3'-primer). The amplified cDNA products are separated on 2% standard agarose gels and stained with ethidium bromide. Primer combinations (5' and 3') are as follows: ACP5, ACP5 + dT-ACP2; ACP16, ACP16 + dT-ACP2; ACP24, ACP24 + dT-ACP2; ACP28, ACP28 + dT-ACP2; ACP34, ACP34 + dT-ACP2; ACP43, ACP43 + dT-ACP2; ACP89, ACP89 + dT-ACP2; ACP113, ACP113 + dT-ACP2. Arrowheads indicate differential cDNA bands (C1–C9) selected due to hatched blastocyst-specific expression. These cDNA bands were excised from the gel for further cloning and sequencing.

employed. Sequence-specific primers were designed to amplify products with lengths ranging from 150 to 288 bp (Table 2). The relative abundance of all samples subjected to real time RT-PCR was calculated using the standard curve method with determination of PCR

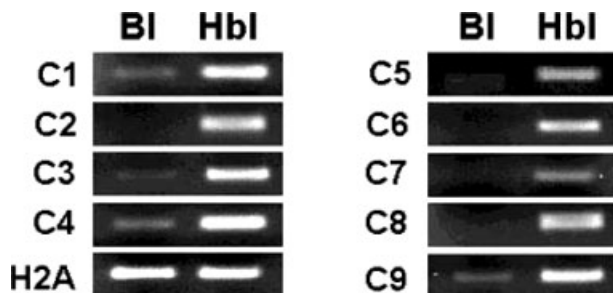


Fig. 3. Differential expression of genes/sequences between two developmental stages. Comparison of the patterns of expression of DEGs (C1–C9) by RT-PCR was performed with embryo-derived RNAs expressed at two developmental stages (Bl; blastocyst, Hbl; hatched blastocyst). The amplified DNA products were separated on a 1.5% standard agarose gel and stained with ethidium bromide. Bovine histone H2a was used as a control to confirm the integrity of the mRNA samples.

amplification efficiency and normalization for histone H2a being used as the internal reference. The expression of this housekeeping gene was assumed not to vary between the different stages (Robert et al., 2002). The real time RT-PCR results are presented as n-fold differences in expression compared to the blastocyst stage, which was considered as a calibrator in this analysis. Quantitative expression patterns of all DEGs and ESTs are presented in Figure 4. This analysis revealed that all of our target transcripts showed expression patterns in agreement with the results of ACP differential display, when their expression at the blastocyst stage was compared to that at the hatched blastocyst stage. Thus, we further confirmed these dynamic expression changes during the early preimplantation period (from a 1-cell embryo to the hatched blastocyst stage) using real time RT-PCR.

Furthermore, the real time RT-PCR analysis allowed us to categorize the target transcripts into two groups on the basis of their expression. RPS12 (C2), PRL12 (C4), AIBP (C5), HDLP (C7), and the COX5a gene (C8) showed similar expression patterns. These transcripts were observed at the 16-cell stage for the first time and progressively increased in expression from the morula, where they were transiently downregulated, to the hatched blastocyst, where maximal expression occurred. RPS12 (C2), PRL12 (C4), AIBP (C5), HDLP (C7), and COX5a gene (C8) transcripts were expressed at 17-, 10-, 16-, 45-, and 7-fold levels at the hatched blastocyst stage, respectively, relative to the blastocyst stage. FTL (C1), LAPTM4a (C3), CULLIN-1 (C6), and RECS1 (C9) also showed similar expression patterns. These transcripts gradually increased from the morula stage up to the hatched blastocyst stage, where their expression reached maximum. FTL (C1), LAPTM4a (C3), CULLIN-1 (C6), and RECS1 (C9) were expressed at a level of 17-, 18-, 9-, and 13-fold at the hatched blastocyst stage, respectively, compared to the blastocyst stage. Also, at the 1-cell, 2-cell, 4-cell, and 8-cell stages, RPS12 (C2), PRL12 (C4), HDLP (C7), COX5a (C8), and RECS1 (C9) were not observed, but FTL (C1), LAPTM4a (C3), AIBP

(C5), and CULLIN-1 (C6) transcripts were observed quantitatively, albeit at low levels.

DISCUSSION

Understanding the molecular mechanisms of early bovine embryonic development requires investigation of gene expression to identify developmentally important genes and to further characterize their expression patterns during bovine preimplantation development. Here, we describe a most accurate and extensive PCR technology controlled by an ACP (Seegene, Seoul, South Korea). This primer has a unique tripartite structure consisting of 3'- and 5'-end distinct portions separated by a regulator. Specifically, the structure of ACPs comprises a) a 3'-end region with a target core nucleotide sequence that substantially complements the template nucleic acid for hybridization; b) a 5'-end region with a nontarget universal nucleotide sequence; and c) a poly (dI) regulator region bridging the 3'- and 5'-end sequences (Fig. 1). Also, this ACP linker prevents annealing of the 5'-end nontarget universal sequence. The ACP-based PCR system facilitates the identification of DEGs from samples with low mRNA levels and without generating false positives (Hwang et al., 2003).

In the present study, we used the ACP-based PCR system to analyze genes that were differentially expressed between mature bovine blastocyst embryos and Hbl produced in vitro. A better knowledge of gene expression patterns during preimplantation would give insights into molecular mechanisms controlling early development as well as understanding events that may be compromising in early embryonic mortality. Using dT-ACP2 (reverse primer) and 120 arbitrary ACPs (forward primer) for PCR amplification, we were able to display thousands of embryonic cDNAs for comparative analysis. Of these, the majority of differentially expressed cDNA bands were conserved in the blastocyst at the two developmental stages (Fig. 2B). Subsequently, nine DEGs were identified, and this was confirmed by RT-PCR assay (Fig. 3). Real time quantitative RT-PCR was used to quantify the stage-specific expression of these transcripts throughout preimplantation development (Fig. 4). The sequence analyses results revealed that all cloned ESTs shared significant sequence similarity with human, mouse, rat, and bovine genes or ESTs available in GenBank (Table 3). A total of nine DEGs exhibited a significantly higher sequence similarity (80–100%) with the coding regions of known genes. A higher degree of sequence similarity was observed between our ESTs and known human, mouse, and rat genes, suggesting that our cDNA clones are most likely the cattle homologs of matching genes in these organisms. Thus, several genes that are differentially expressed in Hbl provide interesting candidates for regulation of preimplantation or implantation during embryogenesis, including those involved in protein synthesis (RPL12; ribosomal protein L12 and RPS12; ribosomal protein S12), metabolism (FTL; ferritin light chain and HDLP; hydroxysteroid dehydrogenase like protein), apoptosis (CULLIN-1; SCF complex protein),

TABLE 3. Sequence Similarity and Characterization of Differentially Expressed Transcripts

Functional role	Identity	Clone	Genbank acc. no.	Homology	
Apoptosis	SCF complex protein (CULLIN-1)	C6	NM_003592	Human 89% (723/808)	
			BC029260	Mouse 87% (629/722)	
			XM_342679	Rat 87% (638/730)	
			BC045445	Zebrafish 81% (257/314)	
Electron transport	Cytochrome-c oxidase subunit 5a (COX5a)	C8	BC024240	Human 90% (438/485)	
			NM_145783	Rat 94% (324/343)	
			BC034302	Mouse 90% (361/398)	
			NM_014713	Human 91% (825/899)	
Intercellular communication	Lysosomal associated protein transmembrane 4 alpha (LAPTM4a)	C3	AK088958	Mouse 89% (821/914)	
			BC063179	Rat 89% (821/914)	
			AB098971	Bovine 99% (447/448)	
			NM_174792	Bovine 99% (748/755)	
Metabolism	Bos taurus ferritin, light polypeptide (FTL)	C1	AF288821	Pig 90% (435/481)	
			D14523	Horse 87% (461/527)	
			BC058820	Human 86% (467/539)	
			BC019840	Mouse 85% (400/468)	
			AY093428	Human 96% (92/95)	
	Protein synthesis	Hydroxysteroid dehydrogenase like protein (HDLP)	C7	BC002211	Mouse 91% (86/94)
				XM_232942	Rat 91% (77/84)
				AB099081	Bovine 100% (464/464)
				X79417	Pig 95% (419/440)
				NM_001016	Human 94% (422/448)
Protein synthesis	Bos taurus ribosomal protein S12 (RPS12)	C2	NM_011295	Mouse 92% (405/437)	
			BC058460	Rat 91% (412/448)	
			AB098784	Bovine 99% (526/527)	
			NG_000962	Human 92% (513/552)	
			BC018321	Mouse 90% (504/557)	
Unclassified	Apolipoprotein A-I binding protein (AIBP)	C5	X53504	Rat 90% (504/557)	
			AJ315849	Human 90% (834/922)	
			XM_215635	Rat 87% (621/713)	
			BC058362	Mouse 87% (608/697)	
			BC026348	Human 86% (816/943)	
Unclassified	Responsive to centrifugal force and shear (RECS1)	C9	AB097685	Mouse 84% (774/920)	
			XM-237311	Rat 83% (477/570)	

electron transport (COX5a; cytochrome-c oxidase subunit 5a), and intercellular communication (LAPTM4a; lysosomal associated protein transmembrane 4 alpha).

Recent advances in molecular technology and in vitro production of bovine embryos have facilitated studies of gene transcription in preimplantation embryos. By employing qualitative or quantitative RT-PCR assays, the expression of more than 100 different genes has been studied (Niemann and Wrenzycki, 2001; Bovine Embryo Gene Collection <http://www.begc.crbr.ulaval.ca/index.html>). The majority of the genes are expressed in a stage-specific manner, showing two major patterns of expression; either they appear after the onset of genomic activity or they are expressed throughout the period before and after the onset of embryonic transcription, indicating maternal and embryonic activity (Niemann and Wrenzycki, 2001). In bovine embryos, embryonic gene activation has definitely occurred by the 8- to 16-cell stage (Camous et al., 1986; King et al., 1988; Frei et al., 1989; Kopecny, 1989). As shown in Figure 4, RPS12, PRL12, AIBP, HDLP, and COX5a transcripts were observed at the 16-cell stage for the first time and progressively increased up to the hatched blastocyst stage, where they reached maximum levels. However, FTL, LAPTM4a, CULLIN-1, and RECS1 transcripts were found to increase gradually from the morula stage to the hatched blastocyst stage, where they

reached a maximum. These differences in expression pattern may reflect the induction of a 16-cell- or morula-specific transcription factor following embryonic gene activation.

Protein synthesis in the early preattachment embryo has been investigated extensively in the recent past (Grealy et al., 1996; Thompson et al., 1996; Kuran et al., 2000). The protein content of in vivo derived preattachment bovine embryos at day 16 has been described (Grealy et al., 1996). The protein content increased 2-fold from the morula to the expanded blastocyst stage followed by a 160-fold increase to the hatched blastocyst stage on day 13. After normalization with the internal control, histone H2a, we observed that the relative expression of ribosomal protein S12 and L12-associated protein synthesis increased by approximately 17- and 10-fold, respectively, in the hatched blastocysts compared with blastocysts (Fig. 4). Ribosomes consist of a small 40S subunit and a large 60S subunit. Together these subunits are composed of 4 RNA species and approximately 80 structurally distinct proteins. Ribosomal S12 is a component of the 40S subunit, and belongs to the S12E family of ribosomal proteins. Increased expression of this gene in colorectal cancers compared to matched normal colonic mucosa has been observed and was identified as a marker for the early detection of human squamous cell carcinoma of the

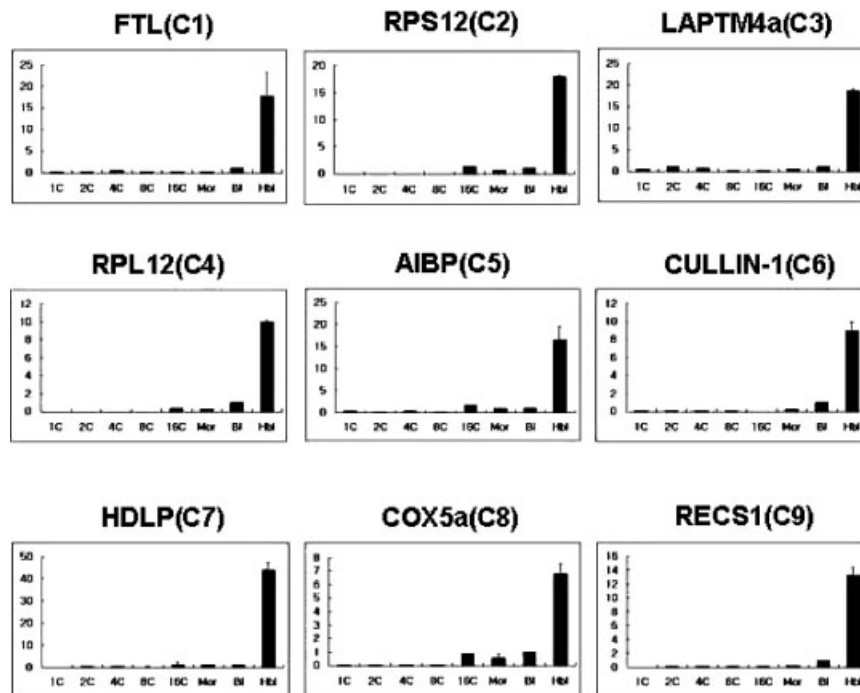


Fig. 4. Relative expression level of DEGs derived from hatched blastocyst embryos throughout the preimplantation stages as quantified by real time RT-PCR. Messenger RNA from pools of 1-cell (1C), 2-cell (2C), 4-cell (4C), 8-cell (8C), 16-cell (16C), morula (Mor), blastocyst (Bl), and hatched blastocyst (Hbl) stages were reverse transcribed and subjected to real time quantitative PCR using transcript-specific

primers (Table 2). All PCRs were conducted in triplicate and normalized for histone H2a mRNA expression. Also, each of these relative values were further divided by the value of the calibrator (blastocyst stage) and the relative expression level is presented as an n-fold expression difference compared to the calibrator, blastocyst (Bl) stage. Data are shown as means + SD (bars) of triplicate determinations.

uterine cervix (Cheng et al., 2002a,b). Ribosomal L12 is a component of the 60S subunit and belongs to the L11P family of ribosomal proteins. Thus, RPS12 and RPL12 may be bifunctional in that they also serve as stage-specific regulators of the hatched blastocyst embryo during early embryogenesis.

Although protein synthesis is critical, proteolysis is equally vital for the upkeep of appropriate levels of short-lived and regulatory proteins that are involved in basic cellular processes such as regulation of the cell cycle and cell division, cell differentiation, metabolism, stress responses, apoptosis, and signal transduction (Schwartz and Ciechanover, 1999; Ciechanover et al., 2000). In eukaryotic cells, ubiquitin-mediated degradation plays a critical role in controlling the turnover of cell cycle regulators (Deshaies, 1999; Tyers and Jorgensen, 2000). The ATP-dependent attachment of ubiquitin to a ubiquitin-activating enzyme (E1) activates ubiquitin for transfer to a ubiquitin-conjugating enzyme (E2) and then to a ubiquitin ligase (E3), which transfers ubiquitin to a substrate protein (Hershko and Ciechanover, 1998; Pichart, 2001). Repetition of this ubiquitin transferase reaction results in the attachment of a polyubiquitin chain to the substrate, which is then recognized by the 26S proteasome and degraded. Cullins are a core component of a subset of E3 ligases. We observed that relative expression of cullin-1 increased by approximately ninefold in the hatched blastocysts compared with blastocysts. Cullin-1 is a component of the SCF complex, which is related to yeast Cds53 and is

implicated in the ubiquitination of p21, p27, and E2F-1 (Yu et al., 1998; Carrano et al., 1999; Marti et al., 1999). To study the role of Cullin-1 in mammalian development, mice deficient for cullin-1 were generated and null embryos and heterozygous cell lines were analyzed (Strohmaier et al., 2001). Cullin-1^{-/-} embryos implant in the uterine wall, but fail to develop past E5.5, and are almost completely resorbed by E7.5. In the mammalian embryo, developmental events at E5.5–6.5 include the onset of gastrulation and an increase in proliferation of a number of specific, newly differentiated tissues. Taken together, these findings lead to the suggestion of a specific role for Cullin-1 in blastocyst hatching, and during posthatching preattachment in the bovine embryo.

Relative expression of FTL (ferritin, light polypeptide) increased by approximately 17-fold in the hatched blastocysts compared with blastocysts (Fig. 4). Ferritin is a multimeric protein, composed of 24 heavy and light subunits that plays a critical role in iron storage and regulation of intracellular iron homeostasis. It has been proposed as a tumor marker (Marcus and Zimberg, 1974), immune suppressor factor (Matzner et al., 1985), and cytokine regulator (Bentwixh et al., 1996) in addition to its basic function as an iron storage protein. Furthermore, p43 placental isoferritin was found to be present at high levels in serum and placental tissue during normal pregnancy and at low levels or below the limits of detection in pregnancy failure (Maymon and Moroz, 1996; Maymon et al., 2000). Thus, expression of

FTL in hatched blastocysts may be important for the maintenance of early pregnancy.

Apolipoprotein A-I (apoA-I) is the major apolipoprotein of high-density lipoproteins (HDL) and plays an important role in the regulation of the stability, lipid transport, and the metabolism of HDL particles. AI-BP (apoA-I interacting protein) was identified using apoA-I (amino acids 25–267) as a bait in a yeast two-hybrid screening system (Ritter et al., 2002). Although AI-BP is a well-known gene, its role during early development is not clear.

In conclusion, we have used a new differential display method, termed the ACP system, to analyze DEGs in bovine blastocysts and hatched blastocysts produced *in vitro*. The genes identified here will provide insights into mechanisms of preimplantation development and early pregnancy in mammals. Future studies will aim to dissect these pathways by using selective gene inactivation techniques, such as RNA interference.

REFERENCES

- Ailenberg M, Silverman M. 2000. Controlled hot start and improved specificity in carrying out PCR utilizing touch-up and loop incorporated primers (TULIPS). *Bio Tech* 29:1018–1024.
- Bentwixh Z, Weizman C, Moroz C, Bar-Yehuda SJ, Kalinkowitch A. 1996. Immune dysregulation in Ethiopian immigrants in Israel: Relevance to helminth infections. *Clin Exp Immunol* 103:239–243.
- Brownie J, Shawcross S, Theaker J, Whitcombe D, Ferrie R, Newton C, Little S. 1997. The elimination of primer-dimer accumulation in PCR. *Nucleic Acids Res* 25:3235–3324.
- Camous S, Kopecny V, Flechon E. 1986. Autoradiographic detection of the earliest stage of [³H]-uridine incorporation into the cow embryo. *Biol Cell* 58:195–200.
- Carrano AC, Eytan E, Hershko A, Pagano M. 1999. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol* 1:193–199.
- Cheng Q, Lau WM, Chew SH, Ho TH, Tay SK, Hui KM. 2002a. Identification of molecular markers for early detection of human squamous cell carcinoma of the uterine cervix. *Br J Cancer* 86:274–281.
- Cheng Q, Lau WM, Tay SK, Chew SH, Ho TH, Hui KM. 2002b. Identification and characterization of genes involved in the carcinogenesis of human squamous cell cervical carcinoma. *Int J Cancer* 98:419–426.
- Ciechanover A, Orian A, Schwartz AL. 2000. The ubiquitin-mediated proteolytic pathway: Mode of action and clinical implications. *J Cell Biochem* 34:40–51.
- Deshais RJ. 1999. SCF and Cullin/RING H2-based ubiquitin ligases. *Annu Rev Cell Dev Biol* 15:435–467.
- Frei RE, Schultz GA, Church RB. 1989. Qualitative and quantitative changes in protein synthesis occur at the 8–16-cell stage of embryogenesis in the cow. *J Reprod Fertil* 86:637–641.
- Grealy M, Diskin MG, Sreenan JM. 1996. Protein content of cattle oocytes and embryos from the two-cell to the elongated blastocyst stage at day 16. *J Reprod Fertil* 107:229–233.
- Hershko A, Ciechanover A. 1998. The ubiquitin system. *Annu Rev Biochem* 67:425–479.
- Hwang IT, Kim YJ, Kim SH, Kwak CI, Gu YY, Chun JY. 2003. Annealing control primer system for improving specificity of PCR amplification. *BioTech* 35:2–6.
- King WA, Niar A, Chartrain I, Betteridge KJ, Guay P. 1988. Nucleolus organized regions and nucleoli in preattachment bovine embryos. *J Reprod Fertil* 82:87–95.
- Kopecny V. 1989. High-resolution autoradiographic studies of comparative nucleogenesis and genome reactivation during early embryogenesis in pig, man and cattle. *Reprod Nutr Dev* 29:589–600.
- Kopecny V, Biggiogera M, Pivko J, Grafenau P, Pavlok A, Malatesta M. 1996. The cell nucleus in early bovine and caprine preimplantation embryos: Fine structural cytochemistry and immunoelectron microscopy. *Eur J Cell Biol* 70:361–372.
- Kuran M, Robinson JJ, Staines ME, McEvoy TG. 2000. Development and *de novo* protein synthetic activity of bovine embryos produced *in vitro* in different culture systems. *Theriogenology* 55:593–606.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402–408.
- Marcus DM, Zimberg N. 1974. Isolation of ferritin from human mammary and pancreatic carcinomas by means of antibody immunoadsorbents. *Arch Biochem Biophys* 162:493–501.
- Marti A, Wirbelauer C, Scheffner M, Krek W. 1999. Interaction between ubiquitin-protein ligase SCF^{SKP2} and E2F-1 underlies the regulation of E2F-1 degradation. *Nat Cell Biol* 1:14–19.
- Matzner Y, Konijn AU, Shlomai Z, Ben-Bassat H. 1985. Differential effect of isolated placental isoferritins on *in vitro* T-lymphocyte function. *Br J Haematol* 59:443–448.
- Maymon R, Moroz C. 1996. Placental isoferritin: A new biomarker from conception to delivery. *Br J Obstet Gynecol* 103:301–305.
- Maymon R, Jauniaux E, Greenwald N, Moroz C. 2000. Localization of p43 placental isoferritin in human maternal-fetal tissue interface. *Am J Obstet Gynecol* 182:670–674.
- McPherson MJ, Moller SG. 2000. BIOS Scientific Publishers, PCR. Oxfordshire, UK.
- Mohan M, Ryder S, Claypool PL, Geisert RD, Malayer JR. 2002. Analysis of gene expression in the bovine blastocyst produced *in vitro* using suppression-subtraction hybridization. *Biol Reprod* 67:447–453.
- Natale DR, Watson AJ. 2002. Characterization of stage-specific and culture sensitive cDNAs during bovine early development by DD-RT PCR. First International Meeting on Mammalian Embryo Genomics. Quebec City, Canada.
- Niemann H, Wrenzycki C. 2001. Alterations of expression of developmentally important genes in preimplantation bovine embryos by *in vitro* culture conditions: Implications for subsequent development. *Theriogenology* 53:21–34.
- Pichart CM. 2001. Mechanisms underlying ubiquitination. *Annu Rev Biochem* 70:503–533.
- Ritter M, Buechler C, Boethcher A, Schmitz S, Madry A, Orso E, Bared SM, Schmiedeknecht G, Baehr CH, Fricker G, Schmitz G. 2002. Cloning and characterization of a novel apolipoprotein A-I binding protein, AI-BP, secreted by cells of the kidney proximal tubules in response to HDL or ApoA-I. *Genomics* 79:693–702.
- Robert C, McGraw S, Massicotte L, Pravetoni M, Gandolfi F, Sirard MA. 2002. Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos. *Biol Reprod* 67:1465–1472.
- Rosenkrans CF Jr, Zeng GQ, McNamara GT, Schoff PK, First NL. 1993. Development of bovine embryos *in vitro* as affected by energy substrates. *Biol Reprod* 49:459–462.
- Rosenkrans CF, First NL. 1994. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes *in vitro*. *J Anim Sci* 72:434–437.
- Saiki RK, Walsh PS, Levenson CH, Erlich HA. 1989. Genetic analysis of amplified DNA with immobilized sequence specific oligonucleotide probes. *Proc Natl Acad Sci USA* 86:6230–6236.
- Schultz RM, Davis W, Stein P, Svoboda P. 1999. Reprogramming of gene expression during preimplantation development. *J Exp Zool* 285:276–282.
- Schwartz AL, Ciechanover A. 1999. The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annu Rev Med* 50:57–74.
- Strohmaier H, Spruck CH, Kaiser P, Won KA, Sangfelt O, Reed SI. 2001. Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. *Nature* 413:316–322.
- Thompson JG, Sherman ANM, Allen NW, McGowan LT, Tervit HR. 1996. Total protein content and protein synthesis within pre-elongation stage bovine embryos. *Mol Reprod Dev* 50:139–145.
- Tyers M, Jorgensen P. 2000. Proteolysis and the cell cycle: With this RING I do thee destroy. *Curr Opin Genet Dev* 10:54–64.
- Yu ZK, Gervais JLM, Zhang H. 1998. Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21CIP1/WAF1 and cyclin D proteins. *Proc Natl Acad Sci USA* 95:11324–11329.