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# Expression pattern of developmentally relevant genes in cattle preimplantation embryos

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## Introduction

With the advent of genetic improvement in dairy cattle for the last 50 years there is a dramatic increase in milk yield associated with a continuous decline in fertility (Lucy 2001). Despite the generally accepted fertilization rate of 90%, large number of embryos lost during the preimplantation period which encompasses the period from fertilization to implantation. The preimplantation developmental period, which occurs with different timing in various species, is marked by many molecular events including maternal to zygotic transition, compaction of morula and the first differentiation of cells into inner cell mass and trophectoderm cells at the blastocyst stage. It is evident that a normal preimplantation development of an embryo relies on the proper genetic programming during preimplantation period starting even before fertilization during gametogenesis. Embryogenomics approaches, a merge of genomics and developmental biology, have shed light on the genetic interplay during this period and identified a number of genes crucially involved in determining the fate a fertilized zygote. During the last decade, nucleotide sequence information arising from various large-scale sequencing projects of the mammalian genomes has significantly increased. From the cDNA sequences deposited in the UniGene database of the National Institutes of Health (NIH), about 15 700 mouse preimplantation genes have been identified (Stanton et al. 2003). The reviewed report by Ko (2004) has shown a total of 140 111 ESTs derived from pre-implantation mouse embryos to be available in the public sequence database (National Center for Biotechnology Information (NCBI) UniGene as of 22 July 2003. Even if it is not with the same pace like in the mouse, the bovine embryonic ESTs derived from various projects are accumulating increasingly (Ko 2004). For this various gene / EST detection methods have been applied to the mammalian preimplantation development including suppression subtractive hybridization (SSH) (Ponsuksili et al. 2002a; Mohan et al. 2002, 2004; Roberts et al. 2000; Yao et al. 2003; Zeng and Schultz 2003), stage specific cDNA libraries (Ponsuksili et al. 2002b, 2001; Adjaye et al. 1997, 1998, 1999; Ko et al. 2000), mRNA differential display (Ponsuksili et al. 2002a; Zimmermann and Schulz 1994; Kanka et al. 2003; Rizos et al. 2002) and serial analysis of gene expression (Neilson et al. 2000; Blomberg and Zuelke 2004; Stanton et al. 2002). Moreover, *in silico* data mining from the existing cDNAs/ESTs has been used to identify genes with crucial functions in preimplantation development (Choo et al. 2001; Hwang et al. 2001; Stanton et al. 2002).

In addition to this the amount of data on the expression pattern of embryonic transcripts in IVP and in vivo-derived bovine embryos has increased significantly. Especially the comparisons of gene expression of bovine preimplantation produced in vitro or in vivo has demonstrated the existing variation in the expression of genes due to the effect of the in vitro culture systems (Niemann and Wrenzycki 2000; Yaseen et al. 2001; Bertolini et al. 2002; Rizos et al. 2002; Tesfaye et al. 2004; Lonergan et al. 2003b). Most of these studies have been carried out under different experimental conditions and relied on the RT-PCR technologies by gene-by-gene approach, which are limited by the number of genes analysed at a time. Since long time ago, it is evident that thousands of genes are involved in preimplantation stage embryo development (Niemann and Wrenzycki 2000). For this, recent developments in cDNA microarray analysis provide the means to measure the expression of tens of thousands of genes in a single experiment. However, due to small amount of biological

material available for analysis in preimplantation expression analysis, cDNA or mRNA amplification procedures are inevitable for such global gene expression analysis. For this purpose, nowadays various global amplification procedures are developed. The main concerns in any mRNA amplification protocols are the representation of transcripts present in the starting material in the final amplified material and the preservation of the relative abundance of transcripts (Adjaye 2005).

Besides identification and expression profiling of developmentally relevant genes, the need to understand the role of these transcripts in preimplantation period is growing from time to time. For this, the recently developed RNA interference (RNAi) technique has emerged as a promising tool to study the function of genes in mammalian preimplantation development (Wianny and Zernicka-Goetz 2000; Svoboda et al. 2000, 2001; Grabarek et al. 2002; Stein et al. 2003; Paradis et al. 2005).

In the present paper we present our results with a review of current advances in gene expression analysis in bovine preimplantation embryos.

**Generation of oocyte or embryonic specific cDNA or ESTs**

Various high-throughput technologies have been utilized to generate mammalian oocyte and preimplantation embryo- specific cDNA/ESTs including differential display (DDRT-PCR) (Ponsuksili et al. 2002; Kanka et al. 2003), suppression subtractive hybridization (SSH) (Zeng and Schultz 2003), serial analysis of gene expression (SAGE) (Velculescu et al. 2003). Moreover, preimplantation stage embryos have been used as source of cDNA/ESTs libraries (Ponsuksili et al. 2002; Ko et al. 2000).

**Stage specific cDNA libraries**

The use of bovine preimplantation embryos as a source of cDNA or ESTs libraries is required for both generation of oocyte or embryo-specific cDNA clones and for profiling of transcripts in various developmental stages of this period. Despite tremendous advances in mouse species, there is still a lack in representation of ESTs from bovine preimplantation embryos. Taking this as a motivation, we have constructed a stage specific cDNA libraries using oocytes, 2-cell, 4-cell, 8-cell, morula and blastocyst stage embryos (Ponsuksili et al. 2002). PCR-based uncloned double-stranded cDNAs have been constructed from the these stages of preimplantation embryos. Moreover, cDNA from the oocyte, 8-cell, morula and blastocyst stage embryos were cloned by ligating into the expression vector TriplEx2 and the cDNA libraries had complexities of $8 \times 10^5$, $5 \times 10^5$, $1 \times 10^6$ and $2 \times 10^6$ independent clones, respectively. From randomly selected and sequenced 48 clones 62.5% (30/48) were homologous to known genes from human and mouse, 18.75% (9/48) to ESTs of human and mouse origin. Novel sequences were identified at a frequency of 14.58% (7/48). The table 1 below showed the BLAST search sequence similarities of the ESTs or cDNA derived from the stage specific libraries to known genes in the public database.

Table 1: Features of some of sequenced clones and results of BLAST search from various libraries

| Library | EST accession no. length (bp) | DNA homology | Accession no. | Identity (%) | Overlap |
|---|---|---|---|---|---|
| Oocyte | BE496795(506) | Human CDC28 protein kinase1 (CKSI) | NM 001826.1 | 93 | 385/411 |
| | BE496797(553) | Human thymosin beta-4 | MI7733.1 | 91 | 358/391 |
| | BE496798(361) | Rattus norvegicus phosphoariginine phoshatase | AB006852 | 92 | 145/156 |
| | BE496802(602) | Human mRNA for KIAAOI97 gene | D83781 | 89 | 427/478 |
| | BE496803(228) | B taurus EST | AW483 146 | 100 | 166/166 |
| | BE496805(287) | Homo sapiens host c factor homolog (LCP) | NM_014315.1 | 93 | 242/259 |

| | | | | | |
|---|---|---|---|---|---|
| | BE496806(1094) | H. sapiens mRNA for putative membrane protein | AB020980.1 | 92 | 537/581 |
| | BE496806(1094) | H. sapiens mRNA for putative membrane protein | AB020980.1 | 92 | 537/581 |
| | BE496807(188) | Novel | | | |
| Eight-cell | BE505072(380) | Human dendritic cell protein (GA57) | NM006360.1 | 91 | 270/296 |
| | BE496797(553) | Human thymosin beta-4 mRNA | M17733.1 | 91 | 358/391 |
| | BE505073(252) | Canis familiaris mRNA for non histone chromosomal protein HMG-17 | AJ388518.1 | 94 | 206/218 |
| | BE505074(437) | B. taurus EST | AW479551 | 98 | 306/311 |
| | BE505076145 | Novel | | | |
| | BE505078(441) | C. familiaris Sec61 complex gamma-subunit | L25086.1 | 94 | 241/254 |
| | BE505080(609) | Novel | | | |
| | BE505081(744) | Pig non histone chromosomal protein (HMG2) | J02895.1 | 92 | 492/534 |
| Morula | BE505053( 240) | Human ribosomal protein S27 (RPS27) | NM001030.1 | 96 | 207/215 |
| | BE505055(357) | Human alpha-NAC mRNA | AF054187 | 95 | 223/234 |
| | BE505057(324) | B. taurus CJ - B13 mRNA for Ubiguinone oxidosereductase complex | X63218.1 | 99 | 320/321 |
| | BE505058(321) | Human ribosomal protein G23 (rPS23) | NM00I025.1 | 92 | 293/317 |
| | BE505059(185) | Human cDNA FL120643 fis | AK000650.1 | 84 | 145/171 |
| | BE505060(443) | Human ribosomal protein S25 (RPS25) | NM001028.l | 92 | 405/439 |
| | BE505064(668) | B. taurus mitochondrion | NC_001567.1 | 99 | 666/668 |
| Blastocytst | BE505086(272) | Novel | | | |
| | BE505087(599) | Novel | | | |
| | BE505088(275) | B. taurus cpn l0 | X69556 | 98 | 261/264 |
| | BE505089(265) | Human testis mitotic checkpoint (BUB3) | AF 047473 | 95 | 243/254 |
| | BE505064(668) | B. taurus mitochondrion | NC001567.1 | 100 | 458/458 |
| | BE505090(306) | Novel | | | |
| | BE505092(593) | Human full length insert cDNA done ZC 48C05 | AF086207.1 | 95 | 568/594 |
| | BE505093(211) | B. taurus EST | AW314583.1 | 96 | 188/194 |
| | BE505094(178) | Homo sapiens TBX3-iso protein(TBX3-iso) | NM_016569.1 | 90 | 86/95 |
| | BE505095(1439) | Human hnRNP core protein Al | X06747.1 | 92 | 1250/135 |
| | BE505096(732) | H. sapiens cDNA FLJIOS9O fis | AK001452.1 | 90 | 396/437 |
| | BE505097 (1257) | H. sapiens TATA box binding protein-(TBP) associated factor | NM_003 187.1 | 93 | 824/883 |

## Suppression subtractive hybridization (SSH)

SSH is used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress nontarget DNA amplification (Diatchenko et al. 1996). This has provided an attractive solution to identify oocyte or embryo specific genes, in particular rare transcripts that may encode regulatory proteins, in various studies (Zeng and Schultz 2003, Robert et al. 2000) including ours (Ponsuksili et al. 2002). The main advantage of this method arises from its ability to normalize the mRNA population so that abundant mRNAs are reduced while rare transcripts enriched. However its disadvantage of this method is that the amount of starting material for hybridization is difficult to obtain from oocytes or embryos. Due to this fact, studies have been carried out using linearly amplified mRNA from mouse oocytes to generate sufficient amounts of material to conduct SSH (Zeng and Schultz 2003). We have conducted SSH using cDNA derived from blastocyst and morula stages used as tester and driver, respectively and vise versa inorder to contract blastocyst enriched

library. The amplification products enriched for blastocyst specific genes were cloned, single clones were randomly picked and insert-DNAs were PCR amplified. Dot blots of the foreward subtracted blastocyst-stage enriched library consisted of 312 clones ranging from 100 bp to 1 kb in length. Differential screening of these clones revealed that 71 clones (22.8%) represent potential target differentially expressed genes. BLASTX searches indicated that from these 71 clones, 26 clones shared homology with known genes and four clones with ESTs and other 3 clones represented novel transcripts. Representative clones and their similarity to the known genes in the public database are indicated in the table2 below.

Table 2: Expressed sequence tags (ESTs) identified from blastocyst-stage transcript enriched cDNA library obtained by SSH

| EST length (bp) | EST Accession number | Methods | Stage | Genes showing similarity with EST[a] | Species | Accession number (BLASTN) | % similarity base pair |
|---|---|---|---|---|---|---|---|
| C25(337) | BQ640947 | Subtractive suppressive cDNA libraries | Blastocyst-stage embryo | Cytochrome oxidase subunit I (10) | *B. taurus* | AF493542.1 | 99% (334/337) |
| C247(223) | BQ640948 | | | Cytochrome oxidase subunit II (3) | *B. taurus* | AF384026.1 | 99% (222/223) |
| C221(454) | BQ640949 | | | Alpha subunit ATP synthase isoform mRNA (2) | *B. taurus* | M22465.1 | 99% (452/454) |
| C31(252) | BQ640951 | | | Lectin, galactoside-binding, soluble, 3 (galectin-3) (6) | *H. sapiens* | BC001120 | 92% (225/242) |
| C245(455) | BQ640952 | | | Fibronectin mRNA (4) | *B. taurus* | K00800.1 | 98% (452/457) |
| C256(307) | BQ640953 | | | Keratin 18 (2) | *H. sapiens* | BC020982 | 89% (249/279) |
| C72(296) | BQ640954 | | | EST (1) | *S. scrofa* | BF704521 | 88% (265/301) |
| C109(176) | BQ640955 | | | Gamma-non-muscle actin (2) | *O.cuniculus* | X60733.1 | 92% (97/105) |
| C110(364) | BQ640956 | | | EST (1) | *B. Taurus* | AW465683 | 97% (321/330) |
| C100(276) | BQ640957 | | | BAC clone RP11-814H16 (1) | *H. sapiens* | AC106052.4 | 94% (260/276) |
| C3(721) | BQ640958 | | | Primary structure of bovine 1.715 satellite DNA (1) | *B. taurus* | V00124.1 | 95% (624/656) |
| C149(376) | BQ640959 | | | BAC clone RP11-814H16 (1) | *H. sapiens* | AC106052.4 | 94% (347/367) |
| C145(235) | BQ640960 | | | Adenylate cyclase-stimulation G-protein alpha subunit (1) | *B. taurus* | X03404.1 | 100% (231/231) |
| C176(239) | BQ640961 | | | Adenine nucleotide translocator 2 (1) | *B. taurus* | AB065433.1 | 99% (226/228) |
| C87(526) | BQ640962 | | | Elongation factor 1 alpha (4) | *B. taurus* | BTA238405 | 99% (523/527) |
| C8(170) | BQ640963 | | | Calpactin I light chain (p11) (1) | *B. taurus* | M16464.1 | 97% (168/172) |
| C154(289) | BQ640964 | | | Calpactin I heavy chain (p36) protein (4) | *B. taurus* | M14056.1 | 98% (286/289) |
| C311(251) | BQ640965 | | | Chaperonin containing TCP1, subunit 3 (gamma) (CCT3) (1) | *H. sapiens* | XM_044127.8 | 94% (234/248) |
| C17(301) | BQ640966 | | | Epithelial cytokeratin (type II) A (7) | *B. taurus* | K03532.1 | 99% (277/279) |
| C7(368) | BQ640967 | | | Annexin A3 (1) | *H. sapiens* | BC000871 | 89% (328/368) |
| C9(279) | BQ640968 | | | Chloride intracellular channel 1 (CLIC1) (1) | *H. sapiens* | NM_001288.3 | 91% (255/279) |
| C24(250) | BQ640969 | | | Frizzled-7 protein - human (LOC151249) (1) | *H. sapiens* | XM_010852.3 | 91% (180/196) |
| C10(160) | BQ640970 | | | Brain protein 44-like (BRP44l) (1) | *H. sapiens* | XM_017634.2 | 88% (122/138) |
| C93(590) | BQ640971 | | | MAPK phosphatase-7 (MKP-7) (1) | *H. sapiens* | XM_039106.3 | 87% (172/197) |
| C78(300) | BQ640972 | | | Novel (1) | | | |
| C89(400) | BQ640973 | | | Novel (1) | | | |
| C91(400) | BQ640974 | | | Novel (1) | | | |
| C180(338) | BQ640975 | | | EST (1) | S. scrofa | BF704036 | 82% (181/220) |
| C141(279) | BQ640976 | | | Mitochondrial phosphate carrier protein precursor (1) | *B. taurus* | X05340.1 | 99% (277/279) |
| C148(320) | BQ640977 | | | Protease, serine, 23 (SPUVE) (1) | *H. sapiens* | NM_007173.2 | 90% (247/272) |
| C33(259) | BQ640978 | | | 70 Kd heat shock cognate protein (5) | *B. taurus* | X53335.1 | 99% (257/259) |
| C239(134) | BQ640979 | | | TERA protein (LOC147363) (1) | *H. sapiens* | XM_006937.7 | 98% (130/132) |
| C76(100) | BQ640980 | | | EST (1) | *S. scrofa* | AU059913 | 98% (98/100) |

**Messenger RNA differential display (DDRT-PCR)**

The preimplantation bovine embryo development is a six-day developmental window in which various events occurred, including the first cleavage division, activation of embryonic genome (Memili et al. 1998), compaction of morula and formation of blastocyst. These

morphological and physiological transitions in preimplantation development of bovine embryos are known to be accompanied and governed by temporal and spatial expression of developmentally important genes (Zimmermann and Schultz 1994; Schultz et al. 1999). It is presumed than thousands of genes need to be expressed in stage-specific manner to achieve normal development of embryos in vitro but to date only 15 physiological functions and the expression of only some genes have been studied (Niemann and Wrenzycki 2000). To better exploit the genes modulating bovine preimplantation development and enrich existing public databank more new developmentally important differentially expressed gene transcripts must be identified and studied in detail. For this several RT-PCR technologies have been applied so far in various species. Of these, the mRNA differential display reverse-transcriptase polymerase chain reaction (DDRT-PCR), which is first developed in 1992 by Liang and Pardee, allowed the identification of stage-specific transcripts including novel and rare transcripts and it has been successfully applied in preimplantation gene expression analysis (Davis et al. 1996; Henrion et al. 1997; Brunet-Simon et al. 2001; Lee et al. 2001; Natale et al. 2000; Goto et al. 2002; Kanka et al. 2003). With the aim of identifying differentially regulated genes during bovine preimplantation development between 8-cells and blastocyts stage embryos, we have conducted mRNA DDRT-PCR using mRNA isolated from 8-cells, 16-cells, morula and blastocyst stage bovine embryos (Tesfaye et al. 2003; Ponsuksili et al. 2002). Using a combination of two base anchored oligo d(T) and 26 random primers more than 900 cDNA bands were conserved in the four developmental stages. The distribution of these bands in the developmental stages showed that 305, 205, 162 and 239 cDNA bands were conserved at 8-cell, 16-cell, morula and blastocyst stages respectively. Analysis of the cDNA bands has indicated the presence of only 6 % (50/911) bands that are differentially expressed. From these, 16 target differentially expressed bands were cloned and sequenced .

Table 3: Embryonic ESTs identified from DDRT-PCR analysis

| EST length (bp) | EST Accession number | Methods | Stage | Genes showing similarity with EST | Species | Accession number (BLASTN) | % similarity base pair |
|---|---|---|---|---|---|---|---|
| 1C9 (234) | BQ640988 | Differential display RT-PCR | 8 cell | Nucleosome assembly protein 1-like 1 (NAP1L1) | *H. sapiens* | NM_139207.1 | 97% (226/232) |
| 1C19 (123) | BQ640990 | | | Novel | | | |
| 1C25 (94) | BQ640991 | | | Cytokine-like nuclear factor n-pac (N-PAC) | *H. sapiens* | XM_048113.3 | 94% (82/87) |
| 1C26.1 (250) | BQ640992 | | | EST | *B. taurus* | BE485962 | 100% (250/250) |
| 1C26.2 (235) | BQ640993 | | | EST | *B. taurus* | BM285439 | 99% (233/235) |
| 1C3 (140) | BQ640994 | | | NY-REN-58 antigen (LOC51134) | *H. sapiens* | NM_016122.1 | 87% (122/140) |
| 1C4 (312) | BQ640995 | | | Pst family repetitive sequence 1 | *B. taurus* | BTRPPST1 | 86% (209/243) |
| 2C2 (405) | BQ640996 | | | Polyadenylate binding protein-interacting protein 1 (PAIP1), | *H. sapiens* | NM_006451.2 | 94% (332/352) |
| 2C9 (201) | BQ640997 | | | Clone IMAGE:3625286, mRNA | *H. sapiens* | BC014103 | 88% (159/179) |
| 4C20 (291) | BQ640998 | | | NADH dehydrogenase subunit 2 | *B. taurus* | AF493541.1\| | 100% (288/288) |
| 1C16 (300) | BQ640989 | | 8 and 16 cell | HMG box transcription factor TCF7L2 (Tcf7l2) | *M. musculus* | AF363726.1 | 91% (233/256) |
| 2C14 (282) | BQ640999 | | | Sec7 and coiled/coil domains 2 (cytohesin-2) (PSCD2) | *H. sapiens* | XM_049963.4 | 91% (256/281) |
| 2C16 (110) | BQ640945 | | 16 cell | EST | *B. taurus* | AV668242 | 99% (108/109) |
| 7C25 (123) | BQ640946 | | | EST | *B. taurus* | BE682976 | 96% (109/113) |
| 2C4 (426) | BQ640944 | | Mor-ula | KIAA1764 protein | *H. sapiens* | BC030701.1 | 89% (223/249) |
| 1C14 (337) | BQ641000 | | Blasto-cyst | EST | *S. scrofa* | AW536053 | 93% (293/313) |
| 8C25 (82) | BQ641001 | | | Novel | | | |

The length of these target sequences ranged from 108 up to 425 bp with the average length of 265 bp. The sequence analysis results showed that all, except one EST, showed significant sequence similarity with known genes or ESTs available in the public database. Nine of the ESTs shared significant sequence similarity (88-100 %) with the coding regions of known genes, while six ESTs were similar with the sequences of known bovine ESTs. Representative number of clones and their sequence similarity with known genes or ESTs in the public data bank are indicated in table 3.

**Expression profiling of transcripts in bovine preimplantation stages**

Besides identification, accurate quantification of gene transcripts is becoming very important for expression characterization of transcripts to get insight into the physiological time table of genes and thereby understanding the molecular mechanisms controlling early embryo development (Steuerwald et al. 1999). For this, the real time quantitative PCR is now emerged as a suitable technique to validate the results SSH, differential display RT-PCR and micro-array experiments (Rajeevan et al. 2001). The physiological association between altered gene expression and abnormal embryo development and quality is not yet fully understood. Therefore, comparative gene expression studies between in vitro or in vivo embryos may enable to study the temporal divergence in the expression of developmentally important transcripts in the two culture systems thereby leading researchers to modify the gene expression patterns through improvement of the in vitro culture system under practice to overcome the problems of viability. Therefore, we have carried out quantitative expression analysis of some selected target transcripts, which are derived from SSH and DDRT-PCR, throughout the preimplantation developmental stages of embryos derived from in vitro or in vivo and with different morphological qualities (Tesfaye et al. 2003, 2004; El-Halawany et al. 2004).

Cumulated evidences are available for the effect of various culture systems on the transcriptional activity (gene expression), quality and developmental rate of embryos in in vitro systems (Wrenzycki et al. 2001; Rizos et al. 2002a, 2002b and 2003; Enright et al. 2000; Lonergan et al. 2003b). Recently it has been demonstrated that, while the oocyte intrinsic quality determines the proportion of oocytes developing to blastocyst (i.e., oocyte developmental competence) (Lonergan et al. 2001), it is the post fertilization culture environment that has the biggest influence on blastocyst quality, irrespective of the origin of the zygote (Rizos et al. 2002a). Moreover, the 6-day postfertilization developmental window is a period in which major developmental events occur, including the first cleavage division, the timing of which is known to be critically important in determining the subsequent development of the embryo (Lonergan et al. 1999); the switching on of the embryonic genome (Memili et al. 1998); compaction of morula and blastocyst formation. There is a clear evidence that any modification of the culture environment in the postfertilization period, which could affect any, or all, of these processes, could have major influence on the quality of the resulting blastocyst (Lonergan et al. 2003b), as measured in terms of cryotolerance and relative abundance of developmentally important transcripts (Rizos et al. 2002a, 2002b, 2002c; 2003).

Representative number of target transcripts derived from SSH and mRNA DDRT-PCR were quantitatively analysed to assess their expression profile through out the preimplantation stages of bovine embryos produced in vitro or in vivo (Tesfaye et al. 2004; El-Halawany et al. 2004). As indicated in the figure 1, transcripts derived from mRNA DDRT-PCR showed different expression profile during a quantitative expression analysis in preimplantation developmental stages of bovine embryos derived in vitro or in vivo.

The PSCD2 gene, which is also named as cytohesin 2 or ARF nucleotide-binding site opener (ARNO), is mapped on chromosome 19 and 18 of human and cattle, respectively. The PSCD2

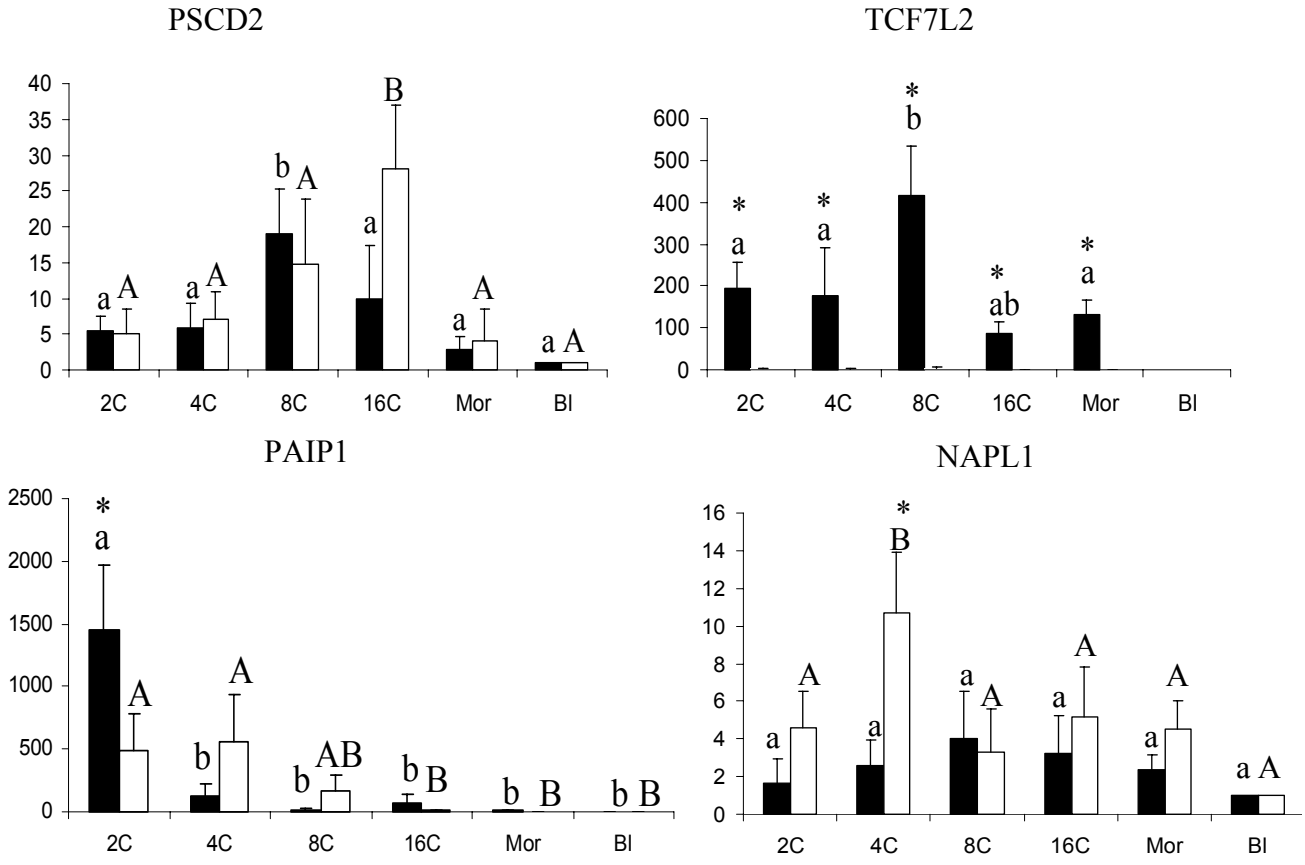


Figure 1: Relative expression profile of transcripts derived from mRNA DDRT-PCR during the postfertilization developmental stages of bovine embryos produced in vitro (black bars) or in vivo (white bars).

gene, as member of the PSCD family has identical structural organization that consists of N-terminal coiled motif, central Sec7 domain and a C-terminal pleckstrin homology domain.
During development polarized traffic pathways are modified to accommodate the specific needs of individual cell types, as well as organization of cells into tissues and organs. Similarly, members of this gene family are known to mediate the regulation of protein sorting and membrane trafficking in cellular physiology (Frank et al. 1998). No significant differences were observed in the level of relative abundance of this transcript between the two embryo groups at any of the developmental stages. However, the relative abundance of this transcript was higher at 8-cell and 16-cell stage among in vitro- and in vivo-derived embryos, respectively.
It is presumed that the current in vitro culture systems can lead to either persistent silencing or enhanced expression of a particular gene through out the critical phases of fetal development. Consistently, in the present study the transcripts TCF7L2 was found to be preferentially expressed in in vitro-produced embryos than their in vivo counterparts. The higher expression level of this transcript in in vitro-produced embryos than in vivo can be considered as a response of the embryo to the suboptimal culture condition or its expression is induced as a result of in vitro culture condition. The high mobility group transcription factor (Tcf) family members are important down-stream effectors of the Wnt signaling cascade in mammalian development and their association with ß-catenin in the nucleus leads to the formation of a transcription factor activating target gene expression (Brantjes et al. 2001). Characterization of members of this gene family in mouse embryos also showed that, *Tcf-3* mRNA is present in embryonic day 6.5 but gradually disappears over the next 3-4 days and *Tcf-4* mRNA expression occurs first at day 10.5 and is restricted to di- and mesencephalon and the

intestinal epithelium during embryogenesis (Korinek et al. 1998). The data of the same experiment showed a direct link between Wnt stimulation and ß-catenin/Tcf transcriptional activation and imply a role for *Tcf-3* and *Tcf-4* in early Wnt-driven developmental decisions in the mouse embryo. In the present study the expression of TCF7L2 gene is found to be specific to in vitro-produced embryos, showing its culture dependent expression pattern.
The PAIP1 gene showed a similar expression pattern in which its relative abundance is higher at 2-cell stage and down-regulated in the later developmental stages in both embryo groups. The relative abundance of this transcript was significantly higher at 2-cell stage in in vitro-produced embryos than their in vivo counterparts. From the expression pattern of the PAIP1 transcript in both embryo groups it is possible to suggest that this transcript is activated from the maternal genome. Overexpression of the PAIP1 gene in COS-7 cells was found to stimulate translation in these cells by providing physical link between the mRNA termini and plays important role in RNA binding and protein synthesis (Craig et al. 1998).
DNA replication has been proposed to provide access for maternally derived transcriptional factors to bind their DNA binding sites and disrupt transcriptionally repressive nucleosomes, both of which result in activation of transcription (Memili and First 1999). The nucleosome assembly protein (NAP) gene family has been implicated in the control of mitotic events (Simon et al. 1994). Its splice variant, NAP1L2 of mouse and human are known to be mainly expressed in nervous system, suggesting an effect on nucleosome assembly or cell-cycle regulation specific to neural function (Rogner et al. 2000). In the present study the NAP1L1 gene was detected through out developmental stages of both in vitro and in vivo embryos with a relatively higher level at 4-cell stage in in vivo-derived embryos than their in vitro counterparts. The relative abundance of this transcript in all of the developmental stages of both embryo groups may suggest its importance in the transcriptional activity of embryos irrespective of whether culture took place in vitro or in vivo.
Some target transcripts derived from SSH experiment were also quantitatively profiled through out the preimplantation developmental stages of in vitro produced bovine embryos (El-Halawany et al. 2004). In addition to confirmation of the results obtained by the SSH, their relative abundance has been investigated as indicated in the figure 2.

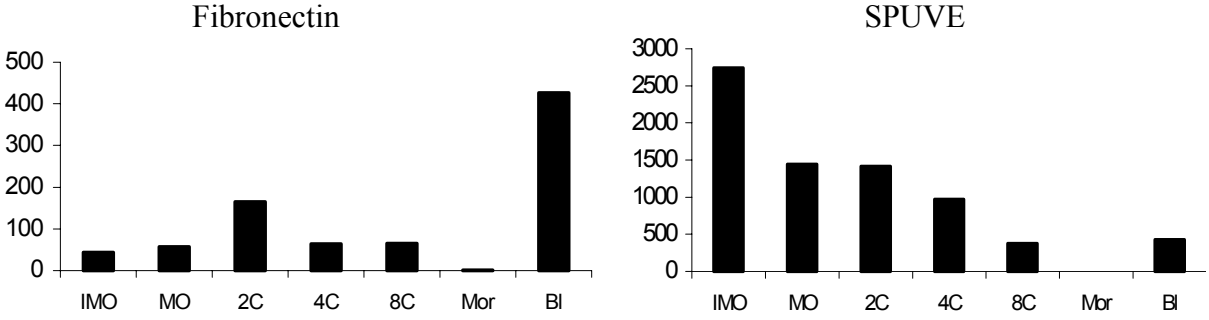


Figure 2: Relative expression level profile of target transcripts (fibronectin and SPUVE) derived from subtractive hybridization through out the preimplantation developmental stages of in vitro produced bovine embryos.

As shown in the figure above the fibronectin transcript is found to be abundant at relatively higher level at the blastocyst stage, which is in agreement with the results of SSH, being moderately abundant at between immature oocytes and the 8-cell stages. However, its relative abundance was drastically down regulated at the morula stages. Fibronectin (FN) forms a fibrillar matrix at the cell surface which controls cell morphology, migration, proliferation and other important cellular processes (Shigemoto 1999, Darribere and Schwarzbauer 2000). The expression pattern of FN in the present study is pertinent to the previous observation by Aplin

et al. (1999) who reported that fibronectin acts as a bridging ligand between the collagen matrix and integrin at the cytotrophoblast surface, mediating anchorage and/or migratory activity which suggest its essential role in implantation process.

The highest expression level of serine protease, 23 (SPUVE) was observed at immature and mature oocyte stages, suggesting the possible involvement of this gene in oocyte maturation and fertilization (for example in sperm-egg interaction) where serine proteases were found to exert their action on matrix degradation (Salamonsen 1999). The expression level is further down regulated till the morula stage and transcript abundance is again upregulated at the blastocyst stage. This up-regulation of this transcript at the blastocyst stage may suggest its role in the process of implantation as it has been reported in the previous reports showing its role in early development in mouse (Teesalu et al. 1996, Chan et al. 1999, O'Sullivan et al. 2001), pig (Geisert et al. 2001) and human (Valdes et al. 2001).

**Global gene expression analysis in bovine preimplantation embryos**

Besides the other high-throughput molecular techniques being developed and applied in preimplantation embryo gene expression analysis (for example mRNA differential display RT-PCR, serial analysis of gene expression, suppression subtractive hybridization and construction of cDNA libraries), microarray is emerging as an efficient method to quickly screen candidate genes related to preimplantation development. DNA microarrays provide the means to measure expression levels of tens of thousands of genes and are usually glass slides or nylon membranes on which DNAs or oligonucleotides are spotted in high density two-dimensional grids. Nowadays various high-throughput cDNA/EST projects provide cDNA clones for the construction of cDNA microarrays for global gene expression analysis in mammalian preimplantation embryos (Sirard et al. 2005; Mamo 2004). For this purpose nowadays many array types are commercially available including in human, mouse and bovine. Before the development of bovine specific arrays, studies have been carried out using heterologous arrays from human to undertake a global gene expression analysis in bovine oocytes (Dalbies-Tran and Mermillod 2003).

The main limitation in the application of microarray technology in embryogenomics is the relatively large amount of RNA input required for hybridization. Usually, 10-100 μg of total RNA or 1-5 μg of mRNA are used as template to create high concentration of the probe in the hybridization buffer and maximize the detection of rare transcripts (Watson et al. 1998; Schena et al. 1995). In contrary to other sensitive molecular technologies where single embryos could be used for gene expression analysis (Ponsuksili et al. 2002; Adjaye et al. 1999), the yield from such single embryos can not be used for microarray analysis with out any form of amplification. For this different probe amplification procedures have been developed and applied in the past. However, the maintenance of the original representation of transcripts in samples is the main concern of any probe amplification procedure (Ivashuta et al. 2002; Cantz et al. 2003). In order to get the optimal RNA amplification protocol without significantly affecting the original representation of transcripts in amplified samples, different amplification cycles were tested with real time PCR (Mamo et al. 2003). Four levels of amplification cycles (10x, 15x, 20x and 25x) were tested by real time amplification of developmentally relevant rate transcripts (RXR-beta and RXR-gama) as shown in figure 3.

With increasing cycle number, the relative abundance and thereby the representation of these transcripts has reduced significantly. Since the 10 times amplification cycle has yielded enough probe material for array hybridization without affecting the representation of rare transcripts, it has been further used for expression analysis of matured oocyte and blastocyst stage embryos using microarray constructed with 82 home produced preimplantation specific genes or ESTs. The analysis results of hybridizations revealed 35 genes and ESTs to be differentially expressed ($p \leq 0.05$). Out of these 24 genes were validated using real-time PCR

assay, of 22 were positively confirmed with their expression pattern observed during array analysis.

a) Relative RXRB expression b) Relative RXRG expression

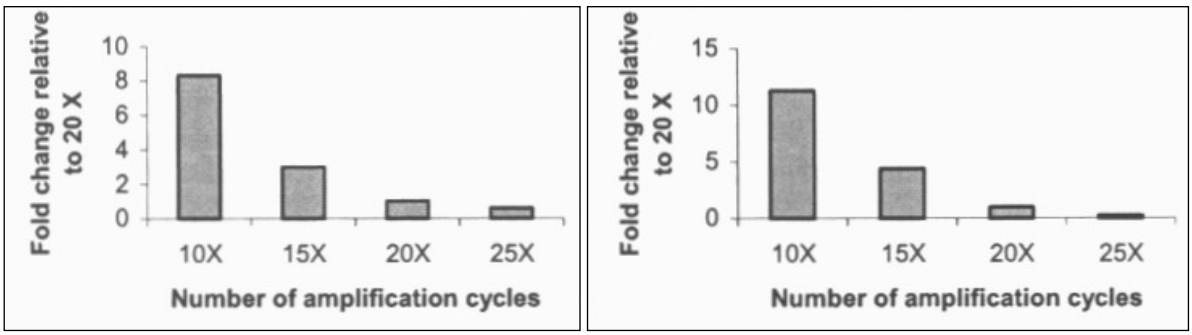


Figure 3: Relative fold change in expression levels relative to the 20 X amplification cicles for (a) RXRB transcripts and (b) RXRG transcripts.

**Gene silencing in preimplantation cattle embryos**

RNA interference (RNAi) has become acknowledged as an effective and useful tool to study gene function in diverse group of cells. This process was first developed for use in invertebrates, later vertebrates, and now after much doubt, has been proved to work for mammals, specifically mice. We aimed to suppress the expression level of the E-cadherin gene during in vitro development of bovine preimplantation embryos using RNAi approach. In this experiment the effect of microinjection of bovine E-cadherin and Oct-4 double-stranded (ds) RNA on the mRNA and protein expression level of the E-cadherin gene was investigated. For this, a 496 bp long bovine E-cadherin (AY508164) and 341 bp long Oct-4 (AY490804) were prepared using Promega RiboMax$^{TM}$ T7 system (Promega, Madison, USA), where sense and antisense strands were transcribed from the target DNA template. In vitro produced bovine zygotes were categorized into four treatment groups (at least 300 zygotes in each group) including those injected with E-cadherin dsRNA, Oct-4 dsRNA, RNase-free water (which was used for resuspension of dsRNA) and non injected control. Statistical analysis was performed using SPSS version 13. While the injection of E-cadherin dsRNA resulted in the reduction of E-cadherin mRNA and protein levels at the morula and blastocysts stage, the same transcript and protein products remained unaffected in the Oct-4 dsRNA, water injected and non treated control groups. The relative abundance of E-cadherin mRNA in the E-cadherin dsRNA injected morula stage embryos was 73% reduced compared to the control group ($P$<0.05). The western blot analysis also showed a significant decrease in the E-cadherin protein (119 kDa) in E-cadherin dsRNA injected embryos compared to the other three groups. Assessment of the effect of the E-cadherin dsRNA on the developmental potential of the embryos showed that both morula and blastocyst rate were lower in E-cadherin dsRNA treated group compared to the other groups. While 22 % of the E-cadherin injected zygotes developed to morula stage, 23 %, 27 % and 38 % of zygotes developed to morula stage in Oct4 dsRNA, water injected and non treated control, respectively. Moreover, 19 % of the E-cadherin injected zygotes developed to blastocyst stage forming blastocoel cavity compared to 17 %, 27 % and 37 % in Oct4 dsRNA, water injected and non treated control, respectively. In conclusion, our results indicated the RNAi technology is a promising approach to suppress the mRNA and protein products of target genes in bovine embryos in order to study their function in early embryogenesis.

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