Sensitivity of bovine blastocyst gene expression patterns to culture environments assessed by differential display RT–PCR

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The use of culture media to support the development of preimplantation embryos to the blastocyst stage is often associated with detrimental effects on normal development. These effects have been uncovered largely by investigating the phenotypic abnormalities displayed by fetuses and newborns derived from cultured preimplantation embryos. Research to understand the impact of culture on the embryonic developmental programme has focused on embryo metabolism, gene expression and genomic imprinting. We have used differential display RT–PCR to examine culture influences on global transcript pools in bovine embryos. Others have examined culture influences on candidate ‘marker genes’ in cultured murine, ovine and bovine embryos. These studies have demonstrated that culture conditions influence the amount of marker gene transcripts and downregulate or induce the expression of novel genes during early development. Optimized defined culture media maintain embryonic gene expression patterns closely resembling those displayed by embryos derived *in vivo*. Preimplantation mammalian embryos display an impressive capacity to respond to the pressures that suboptimal culture environments place upon them. However, this plasticity operates within a defined range of tolerances. Continued research using molecular techniques will lead to increased understanding of developmental mechanisms causing culture-related phenotypic abnormalities in post-implantation embryos.

Steady progress in developing improved culture conditions for preimplantation embryos from a variety of mammalian species has occurred over the past 40 years (Gardner, 1994; Edwards, 1997). The rate of progress has intensified in the last 10–15 years, resulting in the characterization of effective defined or semi-defined media for early embryos in a range of species, including mice (potassium simplex optimized medium, KSOMaa; Ho *et al.*, 1995), hamsters (hamster embryo culture medium 10, HECM-10; Lane *et al.*, 1999), sheep and cows (citrate supplemented synthetic oviduct fluid medium, cSOFMaa; Gardner 1994; Keskintepe *et al.*, 1995; Keskintepe and Brackett, 1996; Walker *et al.*, 1996; Watson *et al.*, 2000), pigs (Machaty *et al.*, 1998) and, most recently, humans (Gardner *et al.*, 1998; Gardner and Schoolcraft, 1999). Preimplantation mammalian embryos display an impressive capacity to respond

positively to the pressures that suboptimal culture conditions place upon them (Ho *et al.*, 1994, 1995; Niemann and Wrenzycki, 2000; Watson *et al.*, 2000). The embryo can (at least partially) compensate for missing components or offset the presence of deleterious components by adjusting its developmental programme (Niemann and Wrenzycki, 2000). However, this plasticity operates within a defined range of tolerances; the vital components of the maternal environment that are required to support early development are yet to be defined, as are their mechanisms and interactions with one another. In addition, current media are known to be suboptimal because cultured embryos from all species (although to a lesser extent in mice) result in reduced pregnancy rates after embryo transfer, and are prone to metabolic and growth disorders that may originate in the culture conditions during preimplantation development (Sinclair *et al.*, 1999; Barker *et al.*, 2000; Boerjan *et al.*, 2000).

These concerns have prompted a great effort to understand how early embryos adjust their developmental programme to compensate for exposure to suboptimal culture environments (for a review, see Niemann and

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Wrenzycki, 2000). Approaches in this research area have included studies comparing expression of different marker genes and also variations in embryo metabolism between preimplantation embryos derived *in vitro* and *in vivo*. We have addressed these concerns by applying differential display (DD)-RT-PCR to assess culture influences on embryonic gene expression patterns. DD-RT-PCR provides an unbiased method to compare mRNA pools from two or more samples (Liang and Pardee, 1992; Zimmerman and Schultz, 1994; DeSousa *et al.*, 1999) and can result in the identification of new gene products. Our objectives were to compare gene expression patterns in bovine blastocysts cultured from the one-cell stage in defined conditions (cSOFMaa and KSOMaa) versus non-defined conditions (TCM-199 + serum and co-culture) and to identify embryonic gene products that may be subject to culture influences. This review summarizes current understanding of culture influences on embryonic gene expression patterns and relates them to our new findings on the global effects of culture environment on embryonic gene expression patterns.

Programme of gene expression during the first week of mammalian development

Estimates of mRNA content in cultured bovine embryos indicate that the content declines between the mature oocyte and the morula stage, and increases again at the blastocyst stage (Bilodeau-Goeseels and Schultz, 1997a,b). This pattern of mRNA loss and re-accumulation mimics the pattern observed in mice, although the increase occurs by the eight-cell stage in mice, possibly as a result of the earlier onset of embryonic gene activation in this species (Piko and Clegg, 1982). DD-RT-PCR revealed the decline in maternal transcripts and renewal of embryonic transcripts that occur after full activation of the bovine embryonic genome (Natale *et al.*, 2000). The first variation from the oogenetic cDNA banding pattern was observed in two- to five-cell bovine embryos. The principal transition from an oogenetic (maternal) to embryonic gene transition occurred at the six- to eight-cell stage. This transition in cDNA banding pattern continued to the blastocyst stage. These transitions in embryonic cDNA banding patterns revealed by DD-RT-PCR are in agreement with the timing of the oogenetic-embryonic transition described for bovine embryos by other investigators (Plante *et al.*, 1994; Viuff *et al.*, 1996, 1998; Memili and First, 1998; Memili *et al.*, 1998). The decreased relative number of oocyte cDNA bands observed throughout bovine pre-attachment development probably reflects the degradation of maternal mRNAs (deadenylation) before activation of the embryonic genome (Paynton *et al.*, 1988; Brevini-Gandolfi *et al.*, 1999).

In bovine embryos, the full activation of the zygotic genome occurs by the 8–16-cell stage as evidenced by [³H]uridine incorporation into nuclei and nucleoli at the eight-cell stage, and major changes in the ultrastructure of blastomere nucleoli and the pattern of protein synthesis (for reviews, see Telford *et al.*, 1990; De Sousa *et al.*, 1998).

Studies investigating [³H]uridine uptake in two- to four-cell embryos revealed that transcriptional activity can be detected earlier than the 8–16-cell stage (Plante *et al.*, 1994; Viuff *et al.*, 1996). Other studies using transcriptional inhibitors and [³H]uridine incorporation into RNA supported these findings (Memili and First, 1998; Memili *et al.*, 1998; Viuff *et al.*, 1998). However, development proceeds to the eight-cell stage in the presence of α -amanitin (Liu and Foote, 1997). These findings are similar to those in other mammalian species (reviewed in Telford *et al.*, 1990; De Sousa *et al.*, 1998). In rabbits, in which the oogenetic-embryonic transition occurs at the 8–16-cell stage, transcriptional activity occurs by the two-cell stage, although the first four cleavages occur in the presence of α -amanitin (reviewed in Telford *et al.*, 1990). Similarly, although oogenetic-embryonic transition is complete in mice by the two-cell stage, zygotic transcription occurs first in male pronuclei (Bouniol *et al.*, 1995; Christians *et al.*, 1995). Extensive studies on early mouse embryos have characterized the degradation of maternal RNAs after fertilization by measuring mRNA de-adenylation (Paynton *et al.*, 1988).

Natale *et al.* (2000) also observed that cDNA banding patterns are largely conserved from the 8–16-cell stage through to the blastocyst stage in bovine embryos. Blastocyst formation is dependent upon transcription up to 84 h after hCG in mice, which is a time of development just preceding blastocyst formation (Kidder and McLachlin, 1985). In bovine embryos, more of the blastocyst mRNAs are detectable first at the 8–16-cell stage and only a comparatively small number of unique mRNAs are present in blastocyst cDNA samples (Natale *et al.*, 2000).

Influence of culture environments on embryonic gene expression

Concerns regarding effects of culture on embryonic gene expression patterns were first raised in studies of early mouse embryos (for a review, see Niemann and Wrenzycki, 2000; Table 1). For example, gene transfection studies using a gene construct encoding the heat shock protein (Hsp) 70.1 coupled to sequences encoding a luciferase reporter gene demonstrated that reporter gene activity is up to 15 times higher in embryos derived *in vitro* than that observed in embryos derived *in vivo* (Christians *et al.*, 1995). Similar approaches have demonstrated that *in vitro* derived embryos produce seven times less platelet activating factor (PAF) than *in vivo* derived embryos (O'Neill, 1997). PAF signalling is crucial for regulating intracellular Ca²⁺, which is a fundamental regulator of cell physiology. Additional studies have examined variations in the expression of mRNAs encoding a wide variety of gene products, including actin, glyceraldehyde-3-phosphate dehydrogenase, Na/K-ATPase, Sp1, TATA box binding protein (TBP), insulin-like growth factor (IGF)-I and -II and IGF-I and -II receptors, in murine preimplantation embryos cultured in KSOMaa or Whitten's medium compared with expression in *in vivo* derived embryos (Ho *et al.*, 1994, 1995). Only a single transcript,

Table 1.
(a) Influence of culture conditions on gene expression in mouse embryos

Gene	Versus <i>in vivo</i>	KSOMaa versus <i>in vivo</i>	Whitten's versus <i>in vivo</i>
Heat shock protein 70.1	15 × increase ^a		
Platelet activating factor	7 × increase ^b		
Actin ^c		NC	NC
Glyceraldehyde-3-phosphate dehydrogenase ^c		NC	Decreased
Na/K-ATPase ^c		NC	NC
Sp1 ^c		NC	NC
TATA box binding protein ^c		Increased	Decreased
Insulin-like growth factor I ^c		NC	Decreased
Insulin-like growth factor II ^c		NC	Decreased
Insulin-like growth factor I receptor ^c		NC	Decreased
Insulin-like growth factor II receptor ^c		NC	Decreased

(b) Influence of culture conditions on gene expression in bovine embryos

Gene	<i>In vitro</i> versus <i>in vivo</i>	Serum versus PVA
Connexin 43 ^d	Decreased	
Desmocollin	NC ^e	Decreased ^f
Plakophilin	NC ^e	Decreased ^f
Glucose transporter I	NC ^e	Decreased ^f
Poly-A-polymerase	NC ^e	Decreased ^f
Heat shock protein 70.1	NC ^e	Decreased ^f
Trophoblast protein	NC ^e	Decreased ^f

^aChristians *et al.*, 1995.

^bO'Neill, 1995.

^cHo *et al.*, 1995.

^{d,e,f}Wrenzycki *et al.*, 1996, 1998, 1999.

KSOMaa: potassium simplex optimized medium; NC: no change; PVA: polyvinyl alcohol.

that encoding TATA box binding protein, was found to vary significantly between the embryos cultured in KSOMaa and those derived *in vivo*. However, eight of these gene products were observed to be present at significantly different amounts in embryos cultured in Whitten's medium compared with either embryos cultured in KSOMaa or embryos derived *in vivo* (Ho *et al.*, 1994, 1995). These studies emphasize that culture environments can influence embryonic gene expression patterns profoundly and that current media, such as KSOMaa, effectively provide the early mouse embryo with an opportunity to express important early genes at normal levels. The influences of culture media on embryonic gene expression patterns do not extend exclusively from the protein or amino acid supplements; ion concentrations can also have important influences on gene expression. Ho *et al.* (1994) compared RNA synthesis and expression of genes from the IGF family in murine embryos cultured in simplex optimized medium composed of either 85 or 125 mmol NaCl l⁻¹. Earlier studies had indicated that 85 mmol NaCl l⁻¹ was linked to higher rates of development *in vitro* and higher rates of protein synthesis (Anbari and Schultz, 1993; Biggers *et al.*, 1993).

Similarly, it was discovered that RNA synthesis rates were reduced by 20% and that RNAs of IGF family members were also reduced significantly in embryos cultured in medium with 125 mmol NaCl l⁻¹ (Ho *et al.*, 1994). Additional studies have reported that these effects of culture on gene expression extend to other gene families such as laminins and glucose transporters (Shim *et al.*, 1996).

Cultured bovine embryos also display a very different pattern of gene expression than do embryos derived *in vivo* (Table 1b). For example, transcripts encoding connexin 43 (Cx43) were detected in *in vivo* derived bovine morulae and blastocysts but not in bovine blastocysts produced *in vitro* and cultured in TCM-199 medium + oestrous cow serum (Wrenzycki *et al.*, 1996). The precise role of Cx43 in mediating early development is still uncertain as Cx43 murine knockouts progress through development to birth without impairment. However, it is clear that gap junctional coupling is a fundamental event that regulates cell-cell communication and thus it is a concern that such an important gene family member is absent in cultured embryos (Niemann and Wrenzycki, 2000). In a second study in which the expression of ten 'marker' genes was

Fig. 1. cDNA banding patterns from a section of a typical differential display (DD)-RT-PCR applied to bovine blastocysts derived from culture of zygotes to the blastocyst stage in two different defined culture media (citrate supplemented synthetic oviduct fluid medium (cSOFM) and potassium simplex optimized medium (KSOM)) and one co-culture/serum supplemented culture system (Co-culture). Boxed regions indicate examples of cDNA bands considered to be differentially expressed. Negative control for DD-RT-PCR is also shown.

Fig. 2. Percentage differences in cDNA banding patterns produced by differential display (DD)-RT-PCR on embryos cultured to the blastocyst stage in TCM-199 + 10% NCS + co-culture (□) versus those cultured to the blastocyst stage in citrate supplemented synthetic oviduct fluid medium (cSOFMaa) or potassium simplex optimized medium (KSOMaa) (■; primers A and B) or those cultured to the blastocyst stage in cSOFMaa (■; primers N, P, Q, R and S). Results represent the number of bands specific to each treatment as a percentage of the average total number of conserved bands counted in triplicate lanes for each primer set (■).

compared between *in vivo* derived bovine embryos and *in vitro* derived embryos cultured in TCM-199 + BSA, only Cx43 was differentially expressed, indicating that cultured bovine blastocysts are deficient in this gene product (Wrenzycki *et al.*, 1998). No other differences in gene expression in these two groups of embryos were reported in this study, which included an examination of desmocollin, plakophilin, glucose transporter 1, polyA polymerase, Hsp 70.1 and trophoblast protein transcripts (Wrenzycki *et al.*, 1998). Another study by the same group reported that supplementing the medium with either serum or polyvinyl alcohol (PVA) influenced the expression patterns of these genes in bovine embryos cultured *in vitro* (Wrenzycki *et al.*, 1999). Only Hsp 70.1 was higher in the serum supplemented group, whereas the PVA supplemented group displayed increased concentrations of the other gene products, especially from the 8–16-cell stage to the blastocyst stage (Wrenzycki *et al.*, 1999). The influences of culture on gene expression patterns can arise as early as oocyte maturation as a significant decline in Na/K-ATPase $\alpha 1$ subunit mRNAs and in cyclin A mRNAs was observed when bovine cumulus–oocytes complexes (COCs) were matured in maturation medium devoid of amino acids (Watson *et al.*, 2000). These effects were also reflected by a reduced developmental frequency to the blastocyst stage by COCs matured in amino acid deficient medium (Watson *et al.*, 2000).

Characterization of culture environment influence on bovine embryonic gene expression by DD-RT-PCR

It has been difficult to measure the full impact of studies that report culture-sensitive influences on embryonic gene expression patterns as, to date, none has been able to couple an analysis of gene expression with an exploration of the consequences to implantation and pregnancy frequencies. This deficiency of data is a result of the extreme technical challenge associated with coupling embryo biopsy and mRNA transcript analysis with embryo transfer studies. However, of greater concern is the absence of a precise list of useful 'marker' genes. As discussed above, only a few genes are influenced by culture environments and, if this approach is to emerge as a practical tool for assessing culture efficacy and embryo health, the list of marker genes must be increased markedly. We have initiated this process by applying DD-RT-PCR analysis to characterize culture-sensitive gene expression patterns in bovine embryos. In our analysis, mRNA pools isolated from blastocysts produced under non-defined (TCM-199 + serum + co-culture) and defined (cSOFMaa and KSOMaa) conditions were screened by DD-RT-PCR using seven different primer combinations.

A section of a typical DD-RT-PCR gel resulting from comparing mRNA profiles of bovine blastocysts cultured in both defined (cSOFMaa and KSOMaa) and non-defined (TCM-199 + serum + co-culture) conditions is shown

Table 2. Sequence screening of differentially expressed cDNAs

Treatment	Size (bp)	Base homology (%)	Sequence similarity
Defined	365	143/159 (89.9%)	AC008908 <i>Homo sapiens</i> chromosome 5 clone CTD-2265E4
Co-culture	260	138/152 (90%)	BTA237934 <i>Bos taurus</i> partial STAT5B gene
		131/146 (89%)	AW313804 9371 MARC 4Bov <i>Bos taurus</i> cDNA
Co-culture	355	55/59 (93%)	C18327 Human placental cDNA
Defined	305	43/44 (97%)	AF008307 <i>Bos taurus</i> neutrophil beta-defensin 4 (BNBD4) gene
Defined	568	168/187 (90%)	AW313642 9044 MARC 1 Bov <i>Bos taurus</i> cDNA
Co-culture	407	284/321 (86%)	AC027313 <i>Homo sapiens</i> chromosome 5 clone CTC-278L1
Co-culture	402	209/249 (84%)	AC010615 <i>Homo sapiens</i> chromosome 19 clone CTD-2561J22

(Fig. 1). In these experiments, the results were analysed by first determining a mean $\pm$ SE for the total bands that were conserved among the triplicate reactions for blastocysts produced under each culture regimen. Each primer set combination defines and amplifies a unique subset of embryonic mRNAs. The total number of cDNAs that a given primer set may amplify is unique but consistent between experimental replicates. The cDNA banding patterns were analysed to determine the number of conserved and unique bands that were present between each blastocyst culture sample and for each primer set combination (Fig. 2). The cDNA banding pattern did not vary between blastocysts produced in the two defined media (cSOFMaa versus KSOMaa). However, when cDNA banding patterns were compared between the defined and non-defined (TCM-199 + co-culture) treatments, primer A revealed five cDNA bands and primer B revealed nine cDNA bands specific to blastocysts cultured under non-defined conditions. In addition, primer A revealed seven specific cDNA bands and primer B revealed six specific cDNA bands in blastocyst samples cultured in defined conditions (cSOFMaa and KSOMaa). This variation in banding pattern between embryos cultured under defined and serum supplemented conditions was also observed when the five additional primer sets were used. However, fewer banding variations were observed with these primer sets. These results indicate that most of the differences in embryonic gene expression patterns will be found between serum-supplemented and serum-free culture conditions. The results also imply that each type of culture environment exerts a unique influence upon the early embryo, resulting in a distinct gene expression pattern for embryos cultured in each medium.

In total, 41 differentially expressed cDNA bands were isolated from this analysis, of which 21 were specific to blastocysts produced under non-defined culture conditions and the remaining 20 cDNA bands were confined to blastocysts produced under defined conditions. The 41 cDNA bands were isolated from a total of 1091 bands that were displayed in all of the samples analysed in this study. This figure represents 3.8% of all cDNA bands displaying an apparent sensitivity to the culture environment.

Differentially expressed mRNAs

It is necessary to clone and sequence each product and confirm its differential expression to proceed further with the analysis of the culture-sensitive gene products. We have initiated this process by sequencing 20 of the 41 differentially expressed cDNA bands. Ten of the products were detected only in the defined media and the remaining ten were observed only in the co-culture non-defined treatment. Of the 20 products, seven displayed a significant homology to cDNA sequences found in Genbank (Table 2). Most of these products represent DNA sequences characterized recently that encode genes of unknown function. Further research is required to determine the expression of these gene products during early development, to confirm their differential expression to specific embryo culture treatments, and subsequently to examine the functional significance of their culture-sensitive gene expression on early development.

In conclusion we have determined that blastocysts cultured under non-defined and defined culture conditions display different mRNA transcript patterns. These differences may not reflect variations in embryo health or developmental potentials but instead may reflect the overall plasticity that the embryo can exhibit to compensate for a suboptimal culture environment. The frequency of blastocyst formation is similar for zygotes placed into either of the culture systems used in the study described. It is predicted that the different patterns of mRNAs displayed by blastocysts cultured in these systems reflect the response of the embryos to different suboptimal environments rather than inferring a decline in overall embryonic health. The embryo may attempt to respond to a suboptimal environment by adjusting its gene expression pattern, and by investigating these responses it may be possible to improve understanding of the genetic programme controlling the first week of development and also to design improved culture media. DD-RT-PCR is an effective method for investigating overall influences on embryonic mRNA pools. Several new putative culture-sensitive embryonic mRNAs have been characterized initially. These products may be of assistance

in contributing to an effective marker gene list once their roles are defined.

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