Gene expression analysis of single preimplantation bovine embryos and the consequence for developmental potential

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Preimplantation embryo development typically involves sequential morphological events connecting embryonic cleavage, morula compaction and blastocyst formation, and occurs in parallel with transcriptional regulation, specifically, the maternal to embryonic transition. The underlying homeostatic and metabolic mechanisms governing embryo development are influenced by both genetic and epigenetic factors that respond to environmental stimuli and may impact development during later gestational and fetal growth. There is a renewed interest in the identification and characterization of developmentally important genes during embryonic and fetal development. Perturbations in gene expression, resulting from environmental conditions, can have serious consequences on further embryonic development, homeostasis and disease pathogenesis. The bovine embryo is, however, capable of tolerating and adapting to a wide range of conditions, although little is known of the molecular fingerprint required for oocyte maturation, fertilization and development to term. The genomic revolution united with promising new technologies offer greater opportunity to elucidate the mechanisms behind this well-orchestrated biological process. This paper reviews the current literature on gene expression in the bovine embryo with reference to environmental interference and the development of new technologies to observe this biological process. Defining the difference in molecular signalling between *in vivo* and *in vitro* systems will undoubtedly improve the safety and efficiency of assisted reproductive technologies. The future challenge is to devise culture conditions that mimic the changing environment required by developing embryos to allow the correct temporal and spatial expression of a cohort of developmental genes in a manner similar to that seen *in vivo*.

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Introduction

The initiation of mammalian embryogenesis is regulated by a complex network involving the oocyte genome, transcriptome and proteome. In the absence of new transcription (Davidson 1986), completion of the first meiotic and mitotic cell cycles, gamete reprogramming (oocyte and sperm) and the maternal to embryonic transition rely on transcripts and proteins made during oocyte growth, as well as signal transduction events associated with maturation, ovulation and fertilization (Knowles *et al.* 2003). During preimplantation development in the mouse, an estimated 15,700 genes are expressed (Stanton *et al.* 2003), and it is likely that a similar number will be expressed in other mammalian species. This preimplantation period culminates in a synchronous and intricate discourse between the embryo and the receptive uterus, resulting in implantation and the pathway to further embryonic development (Wang & Dey 2006).

In vitro production (IVP) technologies provide an alternative source of oocytes and embryos for both research and routine embryo transfer. Historically, IVP success has been primarily gauged on the morphological assessment of the preimplantation embryo. However, while the true developmental competence of any given embryo is a continuum that proceeds throughout its lifecycle, the attainment of full term development is a critical first milestone. This process particularly in a uniparous animal, such as the bovine, is restricted by the long gestational interval and the reliance on intensive recipient management programs, which requires significant capital investment. The relevance is no more apparent then when IVP embryos upon transfer to recipients show similar implantation rates when compared to *in vivo* embryos but then undergo significant embryonic and fetal losses (Reichenbach *et al.* 1992). A proportion (up to 30%) of the surviving animals also show increased birth weight and other anatomical abnormalities that have been described as the large offspring syndrome (Holm *et al.* 1996; Young *et al.* 1998; Renard *et al.* 1999; Niemann & Wrenzycki 2000; Sinclair *et al.* 2000). While bovine preimplantation embryos appear capable of tolerating and adapting to wide ranging environment stimuli, a relative short exposure to sub-optimal *in vitro* conditions can initiate a range of downstream consequences (Wrenzycki *et al.* 2004).

The development of expression analysis techniques to examine the cohort of essential and developmentally important genes during early mammalian development provides a useful method to assess the normality of embryo development and to allow *in vitro* culture and assisted reproductive technologies to be examined and improved without the requirement for extensive *in vivo* testing. The interplay between the effect of microenvironment modifications and epigenetic alternations at early stages of development suggest that limited *in vivo* testing will still be required and not suppressed from future animal studies.

The consequence of temporal or spatial and qualitative or quantitative shifts in gene expression patterns can influence the well-orchestrated events controlling resumption of meiosis, initiation of embryo cleavage, maternal to embryonic transition, and cellular differentiation during and well beyond blastocyst formation.

What is becoming increasingly apparent is that the intrinsic quality of the oocyte plays a key factor in the overall success of these events (Lonergan *et al.* 2003a). However, the molecular fingerprint of an oocyte that is capable of undergoing maturation, fertilization and supporting development to term is virtually unknown.

Defining this profile will likely require comparative gene expression studies involving single embryo analyses, to account for the variability between embryos, with groups of embryos in the same environment where the outcome is to increase the mean behaviour of the group. It would appear however that many of the genes analysed (see Table 1) show consistent expression patterns as a consequence of environmental conditions in both the single and pooled embryo studies. Modifications to culture conditions would necessitate a balance to the envi-

Table 1. Gene expression in bovine oocytes and preimplantation embryos

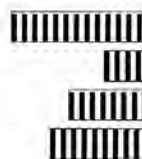| Gene	Expression Profile (Graph Relative Transcript Abundance) in bovine oocytes and embryos									Observations (Single (S) or Pooled (P) oocytes or embryos for analyses)	Reference
	Follicle	Oocyte	Zygote	2-cell	4-cell	8-cell	16-cell	Morula	Blast.		
Acrogranin									+	Aberrant expression in SCNT. Bl Only (S)	(Hall et al. 2005b)
Bax	n.a	+	+	+	+	n.a.	n.a.	n.a.	+	High in poor quality oocyte and embryos (Ref 2, (P)) and (Ref 1, (S))	(Yang & Rajamahendran 2002; Li et al. 2006)
Bcl2	n.a.	+	+	+	+	n.a.	n.a.	n.a.	+	High in good quality oocyte and embryos (Ref 2, (P)) and (Ref 1, (S))	(Yang & Rajamahendran 2002; Li et al. 2006)
bFGF	+	+	+	+	+	+	-	-	+	Up Regulated in IVP Embryo. Bl Only (S)	(Lazzari et al. 2002)
BMP15	+	+	+	+	+	+			-	(P)	(Pennetier et al. 2004)
Cdx2									+	Aberrant expression in SCNT. Bl Only (S)	(Hall et al. 2005b)
Chop-10									+	(S)	(Li et al. 2006)
Cu/Zn-SOD									+	Up Regulated in IVP blastocysts. Bl Only (S)	(Lazzari et al. 2002)
Cx31									+	Bl Only (P)	(Rizos et al. 2004)
Cx43				(graph)	(graph)	(graph)	(graph)			(Ref 1, (P)) and (Ref 2, (S))	(Gutierrez-Adan et al. 2001; Li et al. 2006)
Dc II								+	+	Variable expression in IVP morula and blastocyst (Ref 2, (P) and Ref 1, (S))	(Wrenzycki et al. 2001a ; Knijn et al. 2005)
Dc III								+	+	Up regulation in IVP morula (P)	(Wrenzycki et al. 2001a)
Dlk1	n.a.	-	-	-	-	-		-	-	(S)	(Ruddock et al. 2004)
Dnmt1									+	Up regulation in IVP blastocyst. Bl Only (S)	(Li et al. 2006)
Dnmt3a									+	Gene expression variation according to IVP system. Up regulation in SCNT Blastocysts. Bl Only (P)	(Wrenzycki & Niemann 2003)

Table 1. Contd.

Gene	Expression Profile (Graph Relative Transcript Abundance) in bovine oocytes and embryos									Observations (Single (S) or Pooled (P) oocytes or embryos for analyses)	Reference
	Follicle	Oocyte	Zygote	2-cell	4-cell	8-cell	16-cell	Morula	Blast.		
E-cad								+	+	Decrease in IVP morula (Ref4 (P), Ref 3 (S), Ref 1 (P) and Ref 2 (S))	(Wrenzycki et al. 2001a;b; Rizos et al. 2004; Li et al. 2006)
Eomes									+	Bl Only (S)	(Hall et al. 2005b)
ErbB3									+	Bl Only (S)	(Hall et al. 2005b)
ERR2									+	Aberrant expression in SCNT. Bl Only (S)	(Hall et al. 2005b)
FGF2	n.a.	+	n.a.	+	+	+	n.a.	+	+	(S)	(Daniels et al. 2000; 2001)
FGF4	n.a.	-	n.a.	-	-	-	n.a.	+	+	Decreased expression resulting from SCNT procedure (S)	(Daniels et al. 2000; 2001)
FGFr2	n.a.	+	n.a.	+	+	-	n.a.	-	+	(S)	(Daniels et al. 2000; 2001)
G6PD								+	+	Skewed sex ratio in IVP. Higher expression in Female IVP embryos (S)	(Wrenzycki et al. 2002)
GDF-9	+	+	+	+	+	+	+	-	-	(Ref 1 (P) and Ref 2 (S))	(Pennetier et al. 2004 ; Ruddock et al. 2004)
Glut-1								+	+	Down regulated in IVP morula and blastocyst (Ref 2 (P), Ref 1, 3-4 (S))	(Wrenzycki et al. 2001a; Bertolini et al. 2002; Li et al. 2006; Oliveira et al. 2006)
Glut-3									+	Up regulated expression in IVP Blastocysts. Bl Only (S)	(Lazzari et al. 2002)
Glut-4									+	Up regulated expression in IVP Blastocysts. Bl Only (S)	(Lazzari et al. 2002)
Glut-5										(P)	(Gutierrez-Adan et al. 2001)
Glut-8									+	Up regulated expression in IVP Blastocysts. Bl Only (S)	(Knijn et al. 2005)

Table 1. Contd.

Gene	Expression Profile (Graph Relative Transcript Abundance) in bovine oocytes and embryos									Observations (Single (S) or Pooled (P) oocytes or embryos for analyses)	Reference
	Follicle	Oocyte	Zygote	2-cell	4-cell	8-cell	16-cell	Morula	Blast.		
Gnas	n.a.	−	−	−	−	+	+	+	+	Detected following MET(S)	(Ruddock et al. 2004)
Gp130	n.a.	+	n.a.	+	+	−	n.a.	+	+	Ref 2-3 (S) and Ref 1 (S and P)	(Eckert & Niemann 1998; Daniels et al. 2000; 2001)
Grb10	n.a.	+	+	+	+	+	+	+	+	(S)	(Ruddock et al, 2004)
Hand1									+	Bl Only (S)	(Hall et al. 2005b)
Hsp70.1	n.a.	−	−	−	−	−	−	+	+	Up regulation in IVP and SCNT embryos (S)	(Lazzari et al. 2002; Li et al. 2006)
IFN-ζ	n.a.	−	−	−	−	−	−	+	+	(Ref 2 (S) and Ref 1 (P))	(Rizos et al. 2004; Li et al. 2006)
IGF-1R				(bar graph)	(bar graph)	(bar graph)	(bar graph)		+	(Ref 1 (P) and Ref 2 (S))	(Gutierrez-Adan et al. 2001; Li et al. 2006)
IGF-IIR									+	Down regulated in female IVP blastocysts Bl Only (P)	(Bertolini et al. 2002)
IGF-I									+	Bl Only(P)	(Bertolini et al. 2002)
IGF-II				(bar graph)	(bar graph)	(bar graph)	(bar graph)			Down regulated in IVP blastocysts (Ref 1-2 (P) and Ref 3 (S))	(Gutierrez-Adan et al. 2001; Bertolini et al. 2002; Li et al. 2006)
Il-6	n.a.	−	n.a.	−	−	−	n.a.	+	+	Decreased expression resulting from donor cell and SCNT procedure (S)	(Daniels et al. 2000; 2001)

Table 1. Contd.

Gene	Expression Profile (Graph Relative Transcript Abundance) in bovine oocytes and embryos									Observations (Single (S) or Pooled (P) oocytes or embryos for analyses)	Reference
	Follicle	Oocyte	Zygote	2-cell	4-cell	8-cell	16-cell	Morula	Blast.		
Jam		(graph)	(graph)				(graph)	(graph)	(graph)	Intracellular tight junction implicated in blastocyst formation (S)	(Miller et al. 2003)
Lamin B	−	+	+	+	+	+	+	+	+	GV Only, Not detected in MII (S)	(Hall et al. 2005a)
Lamin A/C	+	+	+	+	n.a.	−	−	+	+	GV Only, Not detected in MII	(Hall et al. 2005a)
LIF	+	+	+	+	+	+	+	+	+	(S) and (P)	(Eckert & Niemann 1998)
LR-ß	+	+	+	+	+	+	+	+	+	(S) and (P)	(Eckert & Niemann 1998)
Mash-2									+	Down regulated in IVP blastocysts, Variable expression in blastocysts from different SCNT methods. Bl Only (S)	(Wrenzycki et al. 2001b)
Mater	+	+	+	+	+	−	−	−	−	(P)	(Pennetier et al. 2004)
Mest (iso 1)	n.a.	−	−	−	−	−	−	−	−	(S)	(Ruddock et al. 2004)
Mest (iso 2)	n.a.	+	+	+	+	+	+	+	+	(S)	(Ruddock et al. 2004)
MnSOD				(graph)	(graph)	(graph)	(graph)			(Ref 1 (P) and Ref 2 (S))	(Gutierrez-Adan et al. 2001; Li et al. 2006)
MRJ	n.a.	−	−	−	−	−	−	−	+	Bl Only (S)	(Hall et al. 2005b)
Ndn	n.a.	+	+	−	−	+	+	+	+	Detected following MET (S)	(Ruddock et al. 2004)
Nnat	n.a.	+	+	+	−	−	−	−	+	Allelic expression differences (S)	(Ruddock et al. 2004)

Table 1. Contd.

Gene	Expression Profile (Graph Relative Transcript Abundance) in bovine oocytes and embryos									Observations (Single (S) or Pooled (P) oocytes or embryos for analyses)	Reference
	Follicle	Oocyte	Zygote	2-cell	4-cell	8-cell	16-cell	Morula	Blast.		
Occludin	▦	▦				▦	▦	▦	▦	Intracellular tight junction implicated in blastocyst formation (S)	(Miller et al. 2003)
Oct4	+	+	+	+	+	+	+	+	+	Expression increase after MET (Ref 1-3) (S)	(Daniels et al. 2000; 2001; Kurosaka et al. 2004)
OOP1	▦	▦		▦	▯					Oocyte-specific marker with two splice variants- specifically expressed in bovine (P)	(Tremblay et al. 2006)
Pan ZO-1	▦	▦				▦	▦	▦	▦	Intracellular tight junction implicated in blastocyst formation (S)	(Miller et al. 2003)
Pan ZO-2	▦	▦				▯	▦	▦	▦	Intracellular tight junction implicated in blastocyst formation (S)	(Miller et al. 2003)

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Gene	Expression Profile (Graph Relative Transcript Abundance) in bovine oocytes and embryos									Observations (Single (S) or Pooled (P) oocytes or embryos for analyses)	Reference
	Follicle	Oocyte	Zygote	2-cell	4-cell	8-cell	16-cell	Morula	Blast.		
PGK								+	+	IVP morula and female embryos transcribe more. IVP blastocysts have higher expression (S)	(Wrenzycki et al. 2002)
Plako								+	+	Down regulated in IVP morula and blastocysts (S)	(Wrenzycki et al. 2001a)
Rex1									+	Bl Only (S)	(Hall et al. 2005b)
Sgce	n.a.	+	+	+	+	+	+	−	−	Potential role in oocyte fertilization and/or cleavage (S)	(Ruddock et al. 2004)
SOX				▮	▮	▮	▮			Higher expression in IVP Blastocysts (P)	(Gutierrez-Adan et al. 2001; Rizos et al. 2002a)
XIAP								+	+	Decreased expression in IVP blastocysts (P)	(Knijn et al. 2005)
Xist	n.a.	−	−	−	−	+	+	+	+	Detected following MZT. Increased expression in SCNT embryos (S)	(Wrenzycki et al. 2002; Ruddock et al. 2004)
ZAR1	▮	▮	▮	▮	▮	▮	▮	▮	▮	Changes in expression around MET (P)	(Brevini et al. 2004)
ZO-1α+	▮	▮				▮	▮	▮	▮	Intracellular tight junction implicated in blastocyst formation (P)	(Brevini et al. 2004; Pennetier et al. 2004)

Table 1. Contd.

Gene	Expression Profile (Graph Relative Transcript Abundance) in bovine oocytes and embryos									Observations (Single (S) or Pooled (P) oocytes or embryos for analyses)	Reference
	Follicle	Oocyte	Zygote	2-cell	4-cell	8-cell	16-cell	Morula	Blast.		
ZO-2β	▦	▦				▦	▦	▦	▦	Intracellular tight junction implicated in blastocyst formation (S)	(Miller et al. 2003)

+ expressed, - not expressed, n.a - not analysed, Bl Only - blastocyst stage examined

ronmental response in the individual embryo against those required for a group of embryos, that's even if correlations between morphological data and gene expression profiling are to be established. Another way to discern this variability would be to analyse gene expression of blastomeres following embryo biopsy. These analyses could be used to modify culture conditions to the single embryo according to how it responds to the environment, although the variability of gene expression between individual (or few) blastomeres is not known.

The continual development of highly sensitive techniques will enable the quantitative profiling of transcriptomes and proteomes, which are necessary to regulate and coordinate events from folliculogenesis to early embryo development and are vital to improving the efficiency of *in vitro* production systems (IVP) and assisted reproductive technologies. To date, research has focussed on embryonic cleavage rates (Lonergan *et al.* 2000; Ward *et al.* 2001; Comizzoli *et al.* 2003; Holm *et al.* 2003), developmental arrest (Yang & Rajamahendran 2002), developmental competence of *in vitro* produced (IVP) embryos (Thompson 1997; Holm & Callesen 1998; Enright *et al.* 2000), embryo manipulation (Wrenzycki *et al.* 2001b), culture (Wrenzycki *et al.* 2001a) and metabolism (Khurana & Niemann 2000; Thompson 2000), embryo genomic activation (De Sousa *et al.* 1998a; Memili & First 2000), embryo sex (Avery *et al.* 1992; Xu *et al.* 1992; Gutierrez-Adan *et al.* 2001), oocyte quality (Lonergan *et al.* 2003a), methylation status (Bourc'his *et al.* 2001), protein synthesis (De Sousa *et al.* 1998b), species differences (Wrenzycki *et al.* 2002), transcript abundance (Watson *et al.* 2000) and the functional organization of the nucleus and nucleolus (remodelling and reprogramming) (Hall *et al.* 2005a ; Corcoran *et al.* 2006).

The degree to which embryo culture or manipulation influences gene expression and the downstream consequences are beginning to be revealed. This review will briefly examine gene expression studies in the bovine preimplantation embryo and the development of new methodologies to elucidate optimal conditions for improving the developmental competence of embryos generated from a variety of IVP systems.

In-Vitro production in the bovine

Despite ongoing improvements, the full potential of the IVP production system remains hampered by the overall quality of the embryo when compared to those derived *in vivo*. This is graphically demonstrated in Fig. 1 where embryo viability until weaning from *in vivo* and *in vitro* production systems are shown from this group over a 5 year period (2000-2005).

Differences between *in vivo* and *in vitro* derived embryos have been reviewed extensively (Thompson 1997; Enright *et al.* 2000; Niemann & Wrenzycki 2000; Lonergan *et al.* 2003a) and the disparity between *in vitro* and *in vivo* embryos can be categorized to either the proportion that reach the blastocyst stage of development or survival following embryo transfer. Around 35% of *in vitro* matured and fertilized (IVM/IVF) oocytes reach the blastocyst stage and around 40% survive to term following transfer when contrasted with over 70% and 60% for the *in vivo* counterparts, respectively (Thompson 1997; Holm & Callesen 1998). Manipulation of IVP embryos for the purpose of Somatic Cell Nuclear Transfer (SCNT) reduces the embryo viability even further (Wrenzycki *et al.* 2004).

The source of variation has been attributed to a variety of causes including the processes of *in vitro* maturation, site of and effect (quality) of sperm at fertilization, chromosome imbalance, polyspermy, variation in pronuclei formation, incorrect morula compaction/ blastocyst formation and sensitivity to cooling and freezing due to elevated lipid content (Thompson 1997; Holm & Callesen 1998; Rizos *et al.* 2002b).
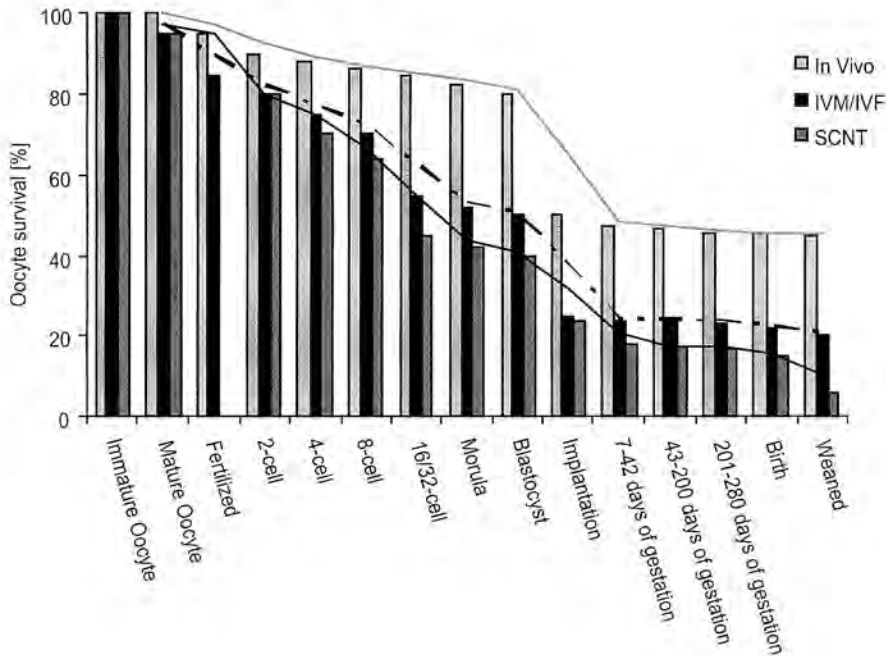
Fig. 1: The developmental competence of bovine embryos derived from *In Vivo* and *In Vitro* production (IVM/IVF and SCNT) systems.

Gene expression analysis in bovine oocytes and embryos

A variety of methods are available for investigating gene expression (see below) and their sensitivity can detect qualitative and quantitative changes to transcriptional regulation during oocyte growth, maturation and embryonic development. Data from these analyses has established molecular expression profiles that may be a more accurate indicator of developmental competence when compared to morphological and blastocyst development observations (Leibfried & First 1979; de Loos *et al.* 1992). The morphological classification and grading of bovine embryos (Lindner & Wright 1983) with minor modifications (Hasler 2001), and subsequently adopted by the International Embryo Transfer (www.iets.org), is the standard to which all embryos are currently described. This practice is used extensively in both research and commercial activities (Thompson 1997; Holm & Callesen 1998; Hyttel 2001). However it has also become increasingly apparent, particularly with the development of SCNT, that morphological data does not correlate solely with developmental competence (see Fig. 1). To battle this discrepancy, much research has recently focused on gene expression profiling in individual oocytes and embryos.

The literature precludes that over 100 genes to date have been associated with developmentally important processes in bovine preimplantation embryos from *in vivo, in vitro* produced, such as genes involved in compaction/cavitation, metabolism, transcription/ translation, DNA methylation and histone modification, oxidative stress, response to or production of growth factors, cytokine signalling, cell cycle regulation and apoptosis. The development of SCNT

technology adds a further level of complexity to gene expression studies where the interrelationship between incomplete somatic cell reprogramming and environmental constraints imposed by the in vitro production system need to be distinguished.

These categories of genes have been evaluated in bovine preimplantation embryos from *in vivo, in vitro* produced and SCNT embryos, for reviews see (Corcoran *et al.* 2005; Niemann & Wrenzycki 2000; Lonergan *et al.* 2001; 2006; Niemann *et al.* 2002; Wrenzycki *et al.* 2004; Wrenzycki *et al.* 2005a;b). Many genes in IVP (and SCNT) embryos have displayed aberrant expression patterns compared with their *in vivo* counterparts. Both genetic and epigenetic mechanisms (methylation and histone modifications) are thought to be involved in the differences in gene expression, regardless of the fact that the developing embryo appears capable of enduring substantial dysregulation of both imprinted and non-imprinted genes (Humpherys *et al.* 2001; Reik *et al.* 2001). There are many steps associated with the IVP system including maturation, fertilization, and culture and in addition to this, various manipulations are undertaken for the production of SCNT embryos, all of which have the potential to further alter gene expression patterns in the developing embryo. These noted alterations in gene expression have been associated with the type of medium (maturation, fertilization and culture) and various additive components (growth factors, serum, etc). Interestingly, epigenetic modifications seem more prevalent in imprinted genes (Blondin *et al.* 2000; Ruddock *et al.* 2004). Many of these differences are clearly established after 1 day of culture, underling the sequential interactions between the environment and gene expression (Rizos *et al.* 2002a;b; 2003; Lonergan *et al.* 2003b). Embryo manipulations involved in the production of SCNT embryos have also resulted in varied gene expression patterns and may be associated with oxidative stress, impaired trophoblastic function, DNA methylation and X chromosome inactivation (Wrenzycki *et al.* 2004). The establishment of diagnostic techniques using these predictive values of aberrantly expressed genes as markers for embryo quality and viability is a critical step towards improving the efficiency of each system. One of the first applications of a limited microarray in the bovine showed 18 genes from a subset of intermediate-filament protein, metabolic, lysosomal-related, stress related and major histocompatibility complex class I were differentially expressed between IVF and SCNT blastocysts. (Gutierrez-Adan *et al.* 2001).

A summary of genes detected in single and pooled bovine oocyte /embryo samples is provided in Table 1. However while a considerable body of work has been amassed on the expression of specific genes and the role of genetic and epigenetic reprogramming, there is a need for greater understanding of the relationships between altered phenotypes, changes in both genetic code and epigenetic patterns within the genome, and alterations in mRNA or protein expression profiles and the downstream consequence for embryos generated from a variety of IVP manipulations. Direct interventions are now required to modify specific epigenetic characteristics to correlate with the biological processes associated with developmental competence, including the correct mRNA and protein expression profiles (Lazzari *et al.* 2002; Fernandez-Gonzalez *et al.* 2004; Farin *et al.* 2006).

Gene expression as an indicator of developmental competence

A number of studies have shown similar rates of ATP production, glucose metabolism, pyruvate uptake and utilization between *in vivo* and *in vitro* produced embryos (Thompson 1997; 2000). However, lower total cell counts in the blastocyst and skewed ratios between inner cell mass and trophectoderm cells in IVP embryos highlight that significant developmental differences exist, which may be reflected at the molecular and cellular level. Gene expression comparisons between *in vivo, in vitro* and SCNT embryos have identified a number of genes associated

with developmental competence. These include the relative abundance of transcripts encoding the α1 subunit of Na1/K1-ATPase (De Sousa et al. 1998c), impaired cavitation and blastocyst formation induced by a decrease in expression of connexin (Cx43) in the IVP blastocyst (Rizos et al. 2002a); absence of FGF4 expression in SCNT blastocysts (Daniels et al. 2000; 2001), altered expression of lamin A/C and B following fusion and early cleavage that was restored by morula stage in SCNT embryos when compared to IVF (Hall et al. 2005a). The developmental competence of oocytes and embryos is influenced by the high expression of Bcl-2 gene (anti-apoptotic) and low expression of the Bax gene (pro-apoptotic), with the ratio of Bcl-2 to Bax expression considered a marker of embryo survival (Yang & Rajamahendran 2002). A recent study examined 16 candidate genes associated with rapid cleavage in bovine two-cell embryos, three genes (YEAF, IDH, H2A) were differentially expressed in the early cleaving embryo and have the potential to be markers of developmental competence (Dode et al. 2006).

The majority of these studies report only steady state mRNA levels in gene expression pathways; however, RNA biogenesis is a central multi-step process that must balance message fidelity against steady-state levels of the mature RNA. An equally important area in the regulation of gene expression in mammals is the role of transcription turnover (Milligan et al. 2002). However, relatively little is known of the consequence of environmental conditions to affect transcription turnover and mRNA half-life leading to altered gene expression pathways in the embryo. Few studies, in this under investigated area, show altered gene expression levels in the embryo are due to changes in transcriptional activity.

Gene expression analysis strategies and DNA amplification

A variety of methods which are suitable for detecting gene transcription in preimplantation embryos are provided in Table 2. Conventionally, methods for detecting gene transcripts have included Northern Blotting, In Situ hybridization and RNAse Protection Assay. Limitations include low overall sensitivity and the need for a high complementary DNA (cDNA) copy number for successful analysis. Given the relative scarcity of mammalian preimplantation embryos and the requirement to pool (100) embryos for sufficient RNA, that is not applicable for analyses of rare constructs or for quantitative measurement in the individual embryo (Lechniak 2002).

Polymerase chain reaction

In contrast, the development of RT–PCR has enabled the detection of mRNAs from low yields of RNA obtained from single embryos (Bustin et al. 2005). However, the requirement to reverse transcribe RNA into cDNA is influenced by a number of variables and even a small shift in amplification efficiency may lead to exponential differences in the final PCR product (Gilliland et al. 1990; Nicoletti & Sassy-Prigent 1996; Lechniak 2002).

The development of competitive (Auboeuf & Vidal 1997) and semi-quantitative, non-competitive RT-PCR (Saric & Sydney 1997) strategies have addressed variability in amplification efficiency. The first involves simultaneous co-amplification of target RNA with added exogenous synthetic RNA that competes with available reagents and primers. The constant ratio between the two types of RNA allows the initial concentration (quantitation) of target RNA to be determined. PCR products are distinguished by small deletions, modifications to restriction sites or the addition of unrelated motifs to the synthetic RNA (Nicoletti & Sassy-Prigent 1996). The second involves co-amplification of the target RNA and unaffected endogenous (GAPDH, ß-actin, rRNA) or exogenous rabbit (ß-globulin) RNA using separate primers. Amplification in

Table 2. Applicable RNA quantitation strategies for the bovine preimplantation embryo

Method	Source	Benefits/ Limitations	Reference
Northern	mRNA	**Ben.:** mRNA size, integrity, alternative splicing **Limit.:** Low sensitivity/ relatively high complementary DNA (cDNA) copy number necessary for successful analysis.	(Bilodeau-Goeseels & Schultz 1997)
RNAse protection assay	mRNA	**Ben.:** Screening of similar size mRNA **Limit.:** Low sensitivity/ relatively high complementary DNA (cDNA) copy number necessary for successful analysis.	(Prediger 2001)
In Situ Hybridisation	mRNA	**Ben.:** Localization of transcript within analysed tissue **Limit. :** Low sensitivity/ relatively high complementary DNA (cDNA) copy number necessary for successful analysis.	(Jin & Lloyd 1997)
RT-PCR	mRNA/cDNA	**Ben.:** Qualitative amplification from a single cell. Semi – quantitative techniques available using competitive and non-competitive RNA. **Limit.:** Variables (primers, polymerase secondary RNA structure) influence efficiency of RT and PCR amplification that do not permit quantitation.	(Bustin et al. 2005)
qRT-PCR	mRNA/cDNA	**Ben.:** multiplex amplification with a single reaction. Quantitation of initial amounts of target sequence. No gel electrophoresis required. **Limit.:** Careful design or primers for amplification process and greater attention to optimization of PCR conditions.	(Gibson et al. 1996) SYBR Green (www.stratgene.com) Molecular Beacons (www.molecular-beacons.org) TaqMan® (www.invitrogen.com)
Microarrays	cDNA arrays	**Ben.:** No sequence analyses required for the array. Product of interest identified by sequence analysis after array. **Limit.:** Requires plasmids from cDNA banks, Difficult maintaining PCR products. Alternative splice variants not distinguished.	(Duggan et al. 1999; Winzeler et al. 1999; Lou et al. 2001)
	Short Oligonucleotides: (25-base) in situ synthesis	**Ben.:** Commercially available. Widely supported. Up to 20,000 genes. **Limit.:** Difficult to incorporate new sequence data	Affymetrix (www.affymetrix.com) Febit (www.febit.de)
	Long (50-80 base) Oligonucleotides	**Ben.:** Design arrays in silico no cDNA banks required. More specificity in hybridization, more constant hybridization. Distinguish between alternative spliced mRNA conditions. Produce arrays with > 25,000 elements. Low and high density arrays Produce arrays with > 25,000 elements. **Limit.:** Requires completion of genome sequence. Design of oligonucleotides is of up most importance.	Combimatrix (www.combimatrix.com) (Blanchard & Hood 1996; Hughes & Shoemaker 2001) Clonetech (http://www.clonetech.com Compugen Http://www.cgen.com MWG Biotech Http://www.mwg-iotech.com
	Genome-Wide SNP:	**Ben.:** 48,000 bovine SNP markers on single array. Near Commercial reality **Limit.:** Reliant on bovine genome sequence. Higher economic cost.	Operon http://www.operon.com Illumina http://www.illumina.com

the linear range for the two primers allows the target RNA PCR products to be normalized against control products (Gutierrez-Adan *et al.* 2000).

A further modification is Real-time PCR which detects mRNA during the exponential phase of amplification and avoids the necessity for subsequent gel analysis (Bustin *et al.* 2005). The assay uses unique florescent reporters (Molecular beacons (Marras *et al.* 2006), SYBR Green (Morrison *et al.* 1998) and TaqMan® probes (Heid *et al.* 1996)), which emit signals in direct proportion to the PCR product during each round of amplification. While care must be taken to optimize both the RNA isolation and RT–PCR conditions, it is possible to detect as few as 400 copies of mRNA (Gibson *et al.* 1996). Internal, exogenous RNA (rabbit globin) and endogenous RNA standards (GAPDH, ß-actin and rRNA) are required during the amplification process to normalize the RNA levels and to justify the observed variations. The application, including use of various primers, detection limitations and benefits of each of the different systems have been described in detail (Lechniak 2002; Wrenzycki *et al.* 2004).

Microarray for analysis of gene expression

The combination and convergence of a variety of technologies led to the development of microarrays in the 1990s. This technology is being continually refined and its widespread adoption has indicated that DNA microarrays are to become the main technological workhorse for gene expression studies (Barrett & Kawasaki 2003; Kunz *et al.* 2004; Taylor *et al.* 2004; Ginsberg 2005). The sensitivity of the assay is high, with reports suggesting that this methodological approach is sensitive enough to detect the presence of one mRNA copy per cell (Barrett & Kawasaki 2003).

The sequencing of the >3 billion nucleotides in the human genome, and its extensive characterization suggest it is comprised of approximately 30,000-40,000 genes. This wealth of genomic information permits researchers to study thousands of genes simultaneously in the cell or tissue type of choice. Subsequently, the (micro) genomic revolution of sequencing is being extended to many different species, including the bovine (see Bovine genome sequencing project paragraph thereafter). Several microarray platforms (see Table 2) are highly suited to the study of gene expression in the bovine preimplantation embryo. Of note are the cDNA arrays (robotic printing), Short Oligonucleotides (25 bases, *in situ* synthesis) and Long Oligonucleotide arrays (40-80 base pairs, *in situ* synthesis and robotic printing) (Barrett & Kawasaki 2003).

The release of the bovine draft sequence and genome assembly has enabled the recent development of high-quality bovine SNP marker arrays to map quantitative trait loci (QTLs) which will result in a tool of significant value to cattle breeding. DNA differences detected through genotyping will expand gene discovery for better meat and milk quality and production, disease susceptibility and the discovery of elements responsible for phenotypic variation, growth and development. With further development it is likely to be applicable to elucidating the relationships between different populations of embryos.

While the application of microarray technologies continues to increase exponentially, it is perhaps cautionary to note that few attempts have been made to replicate and/or compare mRNA data across different platforms. In fact, some comparative cross-platform (and inter-platform) analyses show considerable variations between analyses of the same tissue (Kothapalli *et al.* 2002; Kuo *et al.* 2002). In addition, other methods (RT-PCR etc) have not been used to confirm results. In an effort to address the standardization of both experiments and controls, guidelines have been published to reduce confusion surrounding interpretation and replication of microarray data (Brazma *et al.* 2001; Ball *et al.* 2002; Stoeckert *et al.* 2002).

Bovine genome sequencing project

The bovine sequencing project is an international effort to sequence the genome of the cow (*Bos taurus*). The bovine genome is similar in size to the genomes of humans and other mammals, containing approximately 3 billion DNA base pairs (Gibbs *et al.* 2004).

The collaboration aims to produce a 15-fold coverage of the bovine genome and allow detailed tracking of the DNA differences between a number of cattle breeds to assist discovery of traits for better meat and milk production and to model human disease. The first draft of the bovine genome sequence has been deposited into free public databases for use by biomedical and agricultural researchers around the globe (GenBank (www.ncbi.nih.gov/Genbank) at NIH's National Center for Biotechnology Information (NCBI), EMBL Bank (www.ebi.ac.uk/embl/index.html) at the European Molecular Biology Laboratory's Nucleotide Sequence Database and the DNA Data Bank of Japan (www.ddbj.nig.ac.jp).

Global Gene Expression using cDNA microarray technology

The wealth of sequence data will allow researchers to capitalize on gene expression studies through the flexibility in choosing appropriate and specific arrays (Kurimoto *et al.* 2006; Vige *et al.* 2006). The utility of the microarray platform in preimplantation embryonic development has recently been demonstrated in both the mouse and bovine.

In the mouse, global changes in gene expression during folliculogenesis (primordial to large antral follicles) were examined for pathways that accompany the acquisition of meiotic and developmental competence (Hosack *et al.* 2003). The highest degree of up-regulation and down-regulation of gene expression (one third of transcripts exhibited a two fold change in relative abundance) was observed between the primordial to primary follicle transition (Pan *et al.* 2005). Subsequent transitions were about 10-fold less. Of particular interest in the primordial to primary follicle transition, was the increased or decreased transcriptional activity of specific regions (predominantly) on selected chromosomes. A phenomenon not observed at later stages of oocyte or embryo development.

The changes in global patterns of gene expression during *in vitro* maturation of an oocyte from either a primordial or secondary follicle stage displayed only a 4% and 2% difference when compared to the *in vivo* counterparts, respectively. Additional findings revealed an over-representation of genes involved in transcription of the commonly mis-expressed genes (1%) (Pan *et al.* 2005).

Global patterns of gene expression that surround the development of preimplantation mouse embryos have been examined with cDNA microarrays (Hamatani *et al.* 2004; Rinaudo & Schultz 2004; Zeng *et al.* 2004; Wang S. *et al.* 2005; Zeng & Schultz 2005). Results have confirmed previous analyses showing similarities between oocytes and 1-cell embryos, most likely due to the inheritance of mRNA from the oocyte, with major reprogramming of gene expression associated with maternal to embryonic transition and a period of mid-preimplantation gene activation, which precedes the dynamic morphological and functional changes that occur between the morula and blastocyst stage of development (Hamatani *et al.* 2004). Further analyses during this maternal to embryonic transition revealed a network of genes associated with *Myc* and its role in genome activation and reprogramming of gene expression, and *Hdac1* role in the repression of gene expression through chromatin-mediated changes (Zeng *et al.* 2004; Zeng & Schultz 2005). Global gene expression patterns in preimplantation embryos have been altered by the type of culture medium. Microarray analysis has shown that genes involved in protein synthesis, cell proliferation and transporter function are down-regulated as a consequence of exposure to *in vitro* culture medium (Rinaudo & Schultz 2004; Wang *et al.* 2005).

Microarray analyses have been applied to bovine embryo development (Sirard *et al.* 2005) and recently a 7872 bovine cDNA microarray was use to compare the global gene expression profiles of individual bovine SCNT blastocyst with their donor (somatic) cell and embryos derived from IVF and artificial insemination. The gene expression profile of an individual SCNT blastocyst indicated that it had undergone significant nuclear reprogramming from its original somatic profile (donor cell) so that it unexpectedly resembled a naturally fertilized AI embryo, more so than IVF embryo. Further analysis is required as to whether the consequence of early-stage reprogramming errors (1% of genes examined) have significant downstream effects to embryonic and fetal development (Smith *et al.* 2005). Another more recent study comparing IVF and SCNT blastocysts across 5000 cDNAs could only detect a difference in expression of *KRT18*, and *SLC16A1*. Further examination of transcript levels could not distinguish an NT from an IVF embryo. The unpredictability of gene expression on a global background of multiple gene expression changes argues for a predominantly stochastic nature of reprogramming errors (Somers *et al.* 2006). The dynamic changes during global activation of the embryonic genome have been recently examined using the Affymetrix bovine-specific DNA microarray. In the MII oocytes, genes controlling DNA methylation and metabolism were up-regulated. While during embryonic genomic activation (8-cell), those genes essential to the regulation of transcriptoin, chromatin-structure adhesion and signal transduction were up-regulated. Changes in gene expression during these critical development time points is expected to provide unique chromatin structures that maintain totipotency during embryogenesis and permit lineage-specific differentiation during post-implantation development. The consequence of this dynamics has many implications for a number of assisted reproductive technologies (Misirlioglu *et al.* 2006).

Future developments

While techniques that detect aberrant gene expression at the level of RNA in the single embryo continue to improve, little is known of the consequence at the level of the proteome. There is a need to develop diagnostic non-destructive solutions that permit the selection of viable embryos following *in vitro* culture and assisted reproductive techniques (ie Somatic Cell Nuclear Transfer). Several promising strategies are emerging and their application in cell biology is eagerly awaited. Of particular note is the development of fluorescent techniques that may enable researchers to determine the functions of proteins in individual cells.

Traditionally, florescent organic dyes conjugated with antibodies to the protein of interest have allowed imaging of biological events in individual fixed and permeabilized cells. However, new classes of florescent probes have been developed from synergistic developments in molecular biology, organic chemistry and material science that permit multiple functional analyses (gene expression, protein trafficking, biochemical signals) in single cells using non destructive imaging without significantly perturbing endogenous protein function (Giepmans *et al.* 2006). Two techniques amongst others could be compatible with protein expression studies in the oocyte and developing embryo.

Quantum dots (QD) are inorganic nanocrystals with intense and sustained fluorescence at specific wavelengths. Specialized coatings have made QD water soluble (to prevent quenching) and allow conjugation of protein targeting molecules (antibodies). The final size (QD + biomolecule) prevents easy transport across intact cellular membranes, limiting application to permeabilized cells or extracellular or endocytosed proteins. Further refinements addressing the size limitation using single-molecule optoelectronics have also now been developed (Lee *et al.* 2005).

Genetic Tagging or the construction of genetically encoded fluorophores covalently fused to specific cytoplasmic or surface proteins by spontaneous attachment or enzyme ligation also show potential for biological application. The most developed is the tetracysteine-biarsenical system which modifies a target protein to identify it from the many other proteins inside live cells. This specific protein is then fluorescently stained by membrane permeable non-fluorescent dye molecules that attach with picomolar affinity (Griffin et al. 1998). Small dithiol antidotes are added simultaneously to reduce binding, toxicity and perturbations to endogenous proteins and their function (Martin et al. 2005).

Various modifications to these fluorescent approaches have allowed the study of single proteins or complex endogenous pathways of proteins in a single cell (Sachs et al. 2005), protein localization, diffusion and trafficking (Lidke et al. 2004), dynamic and conformational changes in spatiotemporal resolution (Wallrabe & Periasamy 2005), protein-protein interactions (Gaietta et al. 2002), and by using Chromophore-activated light inactivation (CALI) to inactivate a protein with greater spatiotemporal control than possible with genetic KO or RNA interference approaches (Jay & Sakurai 1999).

Conclusions

In the bovine, as in the mouse and other species, the developmental competence of the preimplantation embryo can be influenced by the environment to which it is exposed. While it is capable of tolerating various sub-optimal conditions, the timing and severity of aberrant gene expression can initiate a range of downstream consequences for fetal and postnatal growth and development. Two critical conditions affect the severity of gene expression. They are the inherent quality of the oocyte which affects its ability to fertilise and form a blastocyst and in vitro culture effects which may impair the quality of the blastocyst. The integration of new and emerging genomic technologies allows researchers to profile the transcription patterns of thousands of genes in single oocytes and embryos following in vitro maturation, fertilization, manipulation and culture. Microarray analyses, when properly standardized, will provide unprecedented levels of information at the molecular level and offer greater opportunity to elucidate the requirements necessary for developmentally competent oocytes and/or embryos. In addition, the availability of new non-invasive florescent technologies that permit the examination of proteins endogenous pathway is eagerly awaited. The potential benefits arising from this knowledge include the ability to select viable embryos using non-invasive, quantitative assays of markers for developmental competence and ultimately the manufacture of in vitro culture conditions that properly mimic the correct temporal and spatial expression of a cohort of developmental genes in a manner similar to that seen in vivo.

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