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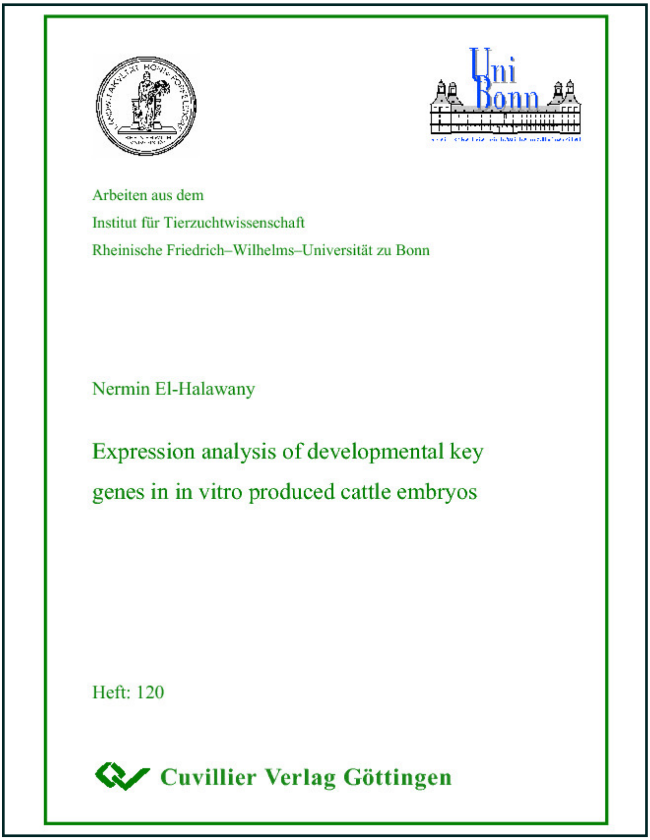

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1 Introduction

In vitro production of bovine embryos (IVP) from immature oocytes offers a commercially viable alternative to breeding programs based on multiple ovulation and embryo transfer techniques for the genetic improvement of live stock. Furthermore, IVP makes an abundant supply of oocytes and embryos of predefined stages available for basic studies on preimplantation bovine development. However, in spite of considerable progress in the recent years in the development of IVP technology, the success rates in terms of embryos yields remain modest and range between 30 % and 40 % (Keskintepe and Brackett 1996, Carolan et al. 1996, Krisher et al. 1999), with approximately 40 % of these being able to initiate pregnancy after transfer (Massip et al. 1995). Contributing to this problem is a lack of basic understanding of gene expression patterns that drive development to the blastocyst stage (Natale et al. 2000). It has been reported that IVP may have profound effects on the genomic activity of preimplantation embryos with potentially severe effects on fetal, prenatal and postnatal viability (Wrenzycki et al. 1996, Bertolini et al. 2002).

Most of the studies directed toward the identification of genes involved in embryo survival and developmental competency have used comparative expression levels of candidate genes. Only a few studies have attempted to compare the entire mRNA content of embryos to find genes associated with developmental competence. Suppression subtraction cDNA library is a feasible and efficient technique for the analysis of stage-specific gene expression in preimplantation development (Morozov et al. 1999). Moreover, the entire task can be accomplished without prior knowledge of genes being expressed and yields subtracted cDNA populations that are either up regulated or differentially expressed (Mohan et al. 2002). In order to pursue a detailed analysis of the processes of early embryonic development, highly sensitive investigative methodologies are required. Real-time PCR which allows monitoring of the accumulation of PCR product at any time point during the amplification reaction enables to solve the major problems that have been inherent to the quantitative PCR in the past (Jung et al. 2000).

Blastocysts usually comprise the most common embryonic stage used for the assessment of embryo production systems, micromanipulations, freezing and transfer to female recipients (Bertolini et al. 2002). Moreover, genes which are differentially

expressed or more abundant in the blastocyst are functional candidates for the regulative processes concerned with the differentiation of ICM and trophoblast cells and the development events taking place in the initial phase of embryo implantation.
Therefore, molecular analysis of the expression profiles of the those genes which are differentiated in blastocyst stage but not in the morula during different preimplantation stages could yield insights into the molecular pathways controlling early development and as a preamble to understand events that may be compromised in early embryonic mortality.
Therefore, the present study was undertaken with the following objectives:

I. Identification of these genes that are switched on during development of morula to blastocyst
II. Analysis the expression levels of those differentiated genes using real-time PCR technology throughout the different stages of preimplantation development, including the critical period at which global embryonic gene activation occurs
III. Compare the expression patterns of the differentiated genes to investigate the onset of transcription for better understanding of the genetic control of this period.

2 Literature review

2.1 Preimplantation development

2.1.1 Oocyte maturation

Mammalian oogenesis begins early in fetal development with the arrival of the primordial germ cells at the developing ovary and the early onset of meiosis at around 10 week gestation in human. However, later in gestation, meiosis is arrested at the diplotene stage of the first meiotic prophase and only resumes in adult reproductive life at puberty (Macklon and Fauser 1999). Resumption of meiosis results from release of an inhibitory effect excreted by follicular cells on oocyte. Upon appropriate stimulation from circulating FSH/LH, final maturation of the oocyte takes place within the mature, preovulatory follicle (Hafez and Hafez 2000a).

2.1.2 Cleavage

After fertilization, embryos enter into several mitotic divisions. The zygote or one –cell stage, is quite large, having a low nuclear to cytoplasmic ratio. To attain a ratio similar to somatic cells, cell divisions occur without an increase in cell mass. This process is referred as cleavage. The resulting daughter cells are called balstomeres. Blastomeres form the two-cell to eight-cell stage in the rabbit and sheep are totipotent, that is fully capable to giving rise to an intact embryo. In four cell embryos no more than three of four blastomeres are totipotent and in eight cell embryos no more than one of eight blastomeres is totipotent (Hafez and Hafez 2000b). However, cloning by nuclear transfer from adult somatic cells (Wilmut et al. 1997) is a remarkable demonstration of developmental plasticity. When a somatic nucleus is placed in oocyte cytoplasm, the changes in chromatin structure that govern differentiation can be reversed and the nucleus can be made to control development to term (Wilmut et al 2002). Several laboratories have used a variety of somatic cell types to create cloned sheep, cattle, mice, pigs, goats, rabbits and cats (results are summarized by Wilmut and Peterson 2002).

2.1.3 Compaction and blastocyst formation

Embryonic compaction is the process of increased cellular flattening and adhesion in 8- to 16-cell embryos that requires the formation of junctional complexes and results in a polarized distribution of cell surface and intercellular component such as the nucleus (Hyafil et al. 1980, Butz and Larue 1995). The process of compaction sets up the initial fate specification of the blastomeres because, after subsequent rounds of mitosis, an embryo has cell with two distinct lineages. The outside cells will become trophoblastic cells, while the blastomeres on the inside of the compacted embryo will form the inner cell mass (ICM) (Johnson and Ziomek 1981, Tam and Behringer 1997). Tight junctions provide a permeable seal that allows fluid to move from outside to the inside of the blastocyst without substantial leakage and from the blastocoel (Hafez and Hafez 2000b). The trophoectoderm ion transport system plays an important role in establishing ion concentration gradients across the epithelium, and thereby in providing the force that drives water into the blastocoelic fluid. Electron probe microanalyses of Na^+, Cl^-, Ca^{2+} and Mg^{2+} have shown that these entire ions are concentrated within the blastocoelic fluid (Borland et al. 1977). The active transport mechanisms required to move these ions against their concentration gradients are thought to involve the transport of Na^+ and Cl^-. The main contributor is the Na, K-ATPase that has been localized to the basolateral domain of the trophoectoderm (reviwed by Agostoni 1993). Blastocyst formation is essential for implantation, establishment of pregnancy and is a principal determination of embryo quality prior to embryo transfer (Watson and Barcroft 2001).

2.1.4 Implantation

Implantation is a critical event, and perhaps the earliest one, in the maternal recognition of pregnancy. Information transfer from conceptus to mother might occur during, and subsequent to, implantation at the level of cell surface interaction (Sherman et al. 1978). At implantation, a highly coordinated process is set into motion whereby specialized cells of the embryo, the trophoectoderm and trophoblast, establish contact with a specialized tissue of the mother, the uterus. The exquisite coordination involves the regulated production of growth factors, cytokines, and hormones by embryonic as well as maternal tissues of both uterine and extrauterine origins. In concert, complementary receptors for these factors must be expressed by the appropriate tissues to propagate implantation signals (Carson et al. 2000). The development of the trophoectoderm and, subsequently, trophoblast creates the embryonic tissue responsible for establishing embryonic contact with the mother. Trophoblast cells, in particular, are quite effective in producing various hormones and cytokines that display profound effects on maternal physiology (Petraglia et al. 1998, Roberts et al. 1999). In addition, trophoblast cells express a number of extracellular matrix receptors and matrix-degrading activities that support interaction with and invasion through the endometrium (Alexander et al. 1996).

2.2 Genetic control of early embryonic development

The molecular control of mammalian preimplantation embryogenesis remains largely unexplored, due mainly to the difficulty of obtaining sufficient quantities of timed embryos for experimentation. Nonetheless knowledge about the changes in gene expression that underlie this period is essential to understand mammalian development (Rothstein et al. 1992).

2.2.1 Expression of maternal genes

A specific complement or abundance of oogenetic mRNAs and proteins is likely required to confer full development competence following fertilization. In mouse, blastocyst formation is dependent on maternal factor(s), present in the egg as mRNA, as well as with zygotic genetic information (Babinet et al. 1990, Renard et al. 1994). During the period of oocyte growth a large number of genes are transcribed and translated. Protein translation actively continues during oocyte maturation and the accumulated transcripts and macromolecules are subsequently used for meiotic and early embryonic developmental events (reviewed by Trounson et al. 2001). During bovine meiotic maturation in vitro, RNA and protein synthesis and phosphorylation of specific oocyte proteins are initially required for germinal vesicle (nuclear) breakdown (GVBD) to occur (Hunter and Moor 1987, Kastrop et al. 1991). Transcription activity in bovine oocytes during folliculogenesis has been reported as early as the secondary follicle stage (Fair et al. 1997). Bovine immature oocytes containing germinal vesicle

(GV) demonstrated transcriptional activity significantly higher than at mature metaphase II, and decrease sharply by the time the oocytes reached arrest (MIII) (Memili et al. 1998). Gene transcripts required for oocyte development have been identified in mouse, these genes as summarized by De Sousa et al. (1998a) include, oocyte specific growth differentiation factor-9 (GDF-9), connexin 37, c-mos proto-oncogene and genes encoding zona pellucida glycoproteins (ZP1, ZP2 and ZP3) which are involved at fertilization and the putative transcription factors controlling their oocyte-specific gene expression.

2.2.2 Genome activation

The activation of embryonic transcription, also known as embryonic gene activation (EGA), represents the beginning of the transition from oogenetic to embryonic control of development. During this transition the embryo begins to synthesize its own mRNA and then protein, replenishing that which it inherited from the mother in the egg, in order to develop beyond early cleavage stage (Schultz 1993). Depending on the species, the EGA has been demonstrated to occur at a certain time after fertilization (Nothias et al. 1995). In mammals EGA appears to commence gradually. In the mouse, rabbit and cow, the species-specific major activation of the genome is in fact preceded by an initial minor degree of embryonic transcription. This activity begins at the end of the first cell cycle in the mouse and rabbit (Kanka et al. 1993, Matsumoto et al. 1994). Studies in the cow have suggested that EGA starts as early as 2-cell stage (Plante et al. 1994, Memili and First 1998, Memili et al. 1998). Recently, bovine 1-cell zygote and 2-cell embryos have found to be transcriptionally and translationally active (Memili and Neal 1999, Hay-Schmidt et al. 2001).

The first round of DNA replication appears to be critical for gene expression in the mouse by disrupting nucleosomes, thereby providing an opportunity for maternally inherited transcription factors to bind to their cognate cis-binding sequences (Wolffe 1994, Davis and Schultz 1997, Memili and Neal 1999).

Another factor that affects gene expression during early development is change in chromatin structure (Wolffe 1996). Turner and Fellow (1989) showed that in mammalian somatic cells, when core histones are acetylated, chromatin loosens and thereby provides an opportunity for transcription initiation factors to be able to bind

DNA. Moreover, deacetylation of histones leads to transcriptional silencing, indicating a direct link between histone acetylation and transcription process (Wolffe and Pruss 1996). Recently, Memili and Neal (1999) reported that acetylation of histones plays an important role in this early gene activation at the onset embryonic development in the cow.

Many genes have been reported to be expressed transiently during EGA, including translation initiation factor eIF-1A (formerly known as eIF-4C) (Davis et al. 1996, De Sousa et al. 1998b), HSP70.1, a member of the multigenic HSP 70 family of heat shock proteins (Bensaude et al. 1983, Christians et al. 1995, Thompson et al. 1995a) and U2afbp-rs, the mouse homologue of the human U2af 35 kDa mRNA splicing factor (Latham et al. 1995).

2.2.3 Genetic control of cleavage and blastocyst formation

Estimates of total RNA content in vitro derived bovine embryos indicated that it declines from the mature oocyte to the morula stage, only to increase again at the blastocyst stage (Bilodeau-Goeseels and Schultz 1997a). The timing and the increase in abundance of specific mRNA transcripts occurs in a transcript specific manner. Examples of change in steady state levels of various mRNA representing both nuclear and mitochondrial gene expression determined by Northern blot analysis (Bilodeau-Goeseels and Schultz 1997b). The existence of many stage-specific expressed genes has two primary implications. First, development processes may lead to significant differences in gene expression in the transition from 2 cells to 8 cells, even though the process appears to be simply a division of cells. Second, stage-specific expressed genes may actively promote the advancement of embryos from one stage to the next. Considering the rapid and selective turnover of these particular mRNAs, there is likely to be selection for their rapid degradation. The requirement of function of that particular gene is apparently transient, which suggest a hit-and-run type of mechanism for expression of cascades of genes (Ko et al. 2000).

Although all zygotically derived gene products are likely to play a role in supporting preattachment development, several gene families have been identified as important contributors. These genes include: