Increasing cloning efficiencies requires a better understanding of developmental abnormalities and gene expression in manipulated embryos

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ABSTRACT: Cloning is a platform technology that will be used to make major advances in agricultural sciences. The result is a more efficient method of making transgenic embryos, fetuses, and offspring. Enhanced production traits and disease resistance may be realized in animal agricultural by utilizing new assisted reproductive technologies. Cloning alone allows for the multiplication of genetically superior adult animals. This multiplication may reduce the need for expensive multiplier herds and allow producers to enhance their products through improved access to elite genetics, while producing a more consistent product that more closely meets and responds to consumer demands. The key development needed to move the technology to larger markets is a more efficient and consistent cloning method. Most problematic are the pregnancy losses and poorer offspring survivability associated with cloning. These losses and abnormalities seem to be at least associated with, but may be caused by, placental abnormalities. Currently, gene products are being isolated from fertilized and cloned embryos. A two-pronged approach is contemplated. One is to determine "normal" and "abnormal" gene expression patterns in embryos and use it as a diagnostic tool to preselect embryos before transfer into recipient animals. The other is to find candidate genes to modify in order to achieve normal development. These problems must be solved or largely negated before anyone can produce thousands of cloned animals. However, for transgenic applications, cloning needs are different. Producing a limited number of cloned transgenic animals is now more efficient than traditional microinjection procedures for production of transgenic animals. An additional benefit for transgenics through cloning is the ability to knock out genes.

Key Words: Gene Expression, Transgenics

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INTRODUCTION

Procedures used in nuclear transfer (cloning) and in vitro embryo production contribute to the various anomalies in embryos and offspring (Stice et al., 1996; Walker et al., 1996). High incidence of posttransfer embryo mortality is a common feature for in vitro-produced (**IVP**) embryos. A post-d-35 mortality rate of 20 to 25% is very high and disturbing when compared to 5% for in vivo-derived embryos (Hasler et al., 1995). In many cases, these early embryo losses have been linked to failures and abnormalities in placentation and embryo vascularization (Peterson and McMillan, 1998). The same placental abnormalities have been observed in the nuclear transfer embryos derived from embryonic cell lines; however, in this case they were more severe, because all embryos died by d 55 of gestation (Stice et al., 1996). Later, somatic cells used in the cattle cloning procedure resulted in offspring, but many of the embryonic, fetal, and neonatal losses were also observed in these studies (Hill et al., 1999).

Developmental Anomalies

It is clear that many of the abnormalities seen in fetuses and placentae are the result of an early event in development. These are commonly referred to as the "large lamb/calf" syndrome (Walker et al., 1992; Behboodi et al., 1995). However, there is a whole array of other developmental pathologies such as abnormal limb and organ development (Willadsen et al., 1991; Farin and Farin, 1995; Sinclair et al., 1997), high neonatal mortality (Massip et al., 1996), and hydroallantois (Willadsen et al., 1991). Wilson et al. (1995) reported an approximately 20% increase in birth weight of cloned calves in comparison to calves of similar genetics produced by embryo transfer or natural mating. Placentae of bovine fetuses derived from IVP embryos had fewer placentomes than those from in vivo embryos (Farin and Farin, 1995). In nuclear transfer bovine embryos, in addition to pathological changes in placentomes, car-

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diopulmonary abnormalities were observed (Stice et al., 1996; Hill et al., 1999).

Culture conditions used during in vitro embryo production may partly contribute to the observed anomalies. In particular, inclusion of serum as a supplement to culture medium during development of sheep embryos has led to oversized lambs (Thompson et al., 1995). Also, the use of coculture systems in both sheep and cattle led to the production of large offspring (Farin and Farin, 1997; Sinclair et al., 1997). Among other factors, ammonia toxicity has been indicated as a factor in fetal oversize (McEvoy et al., 1999). In mice, ammonia toxicity during in vitro culture caused altered patterns of fetal development and reduced viability (Lane and Gardner, 1994).

The above information indicates that in vitro conditions and manipulations can result in anomalies observed later in gestation. Whether these events are a result of altered early gene expression in these embryos is debatable. Therefore, it is prudent to determine first the range of altered gene expression in various types of embryos (in vivo, IVP, and nuclear transfer). This may lead to the identification of new genes involved in the control of early embryonic development, and in future studies, elucidate the molecular basis of observed anomalies in fetuses and placentae. Based on such findings, the existing procedures and protocols may be modified to better mimic the in vivo condition and increase the efficiency of in vitro embryo production and cloning. Ultimately, identified genes may serve as prognostic and diagnostic markers for embryonic viability.

Bovine Embryonic Gene Expression

Differences in gene expression may occur soon after the onset of transcription of the embryonic genome. Interestingly, it has been thought that transition from maternal to embryonic control in the bovine begins at the 8- to 16-cell embryo (Kopecny et al., 1989; Barnes and First, 1991). However, the most recent evidence indicates that bovine zygotes and two-cell embryos are transcriptionally active (Viuff et al., 1996; Memili and First, 1999). These findings suggest that after exposure to conditions in vitro, differences in gene expression may even be seen immediately after fertilization.

Expression of certain genes can be linked to the morphological and functional differences observed between embryos produced in vitro and in vivo. For instance, mRNA for connexin 43 (protein forming gap junctions) has been found only in in vivo morulae and blastocysts (Wrenzycki et al., 1996). This coincides with the findings that gap junction-like structures were functional in inner cell mass (**ICM**) and trophoblast cells (**TE**) of in vivo-derived blastocysts in contrast to blastocyst of IVP bovine embryos (Boni et al., 1999). Reduced expression of gap junctions in IVP embryos may explain the absence of compaction and lower developmental competence of in vitro bovine embryos. Also, the relative abundance of transcripts for the $\alpha 1$ subunit of Na^{+}/K^{+}-ATPase has been linked to oocytes developmental competence (De Sousa et al., 1998). Analysis of the expression of bovine leukemia inhibitory factor (bLIF) and LIF receptor-β genes revealed that these genes were expressed by IVP bovine embryos, but not in their in vivo counterparts (Eckert and Niemann, 1998). The observed differences in gene expression pattern between IVP and in vivo embryos can also result from aberration in methylation pattern of CpG sequences in the gene promoter region, which are known to be essential for gene silencing (Jones, 1999). It is known that methylation is involved in imprinting, and many imprinted genes such as IGF-II, IGF-IIr are important for control of embryonic and placental development (Mann et al., 1995).

Currently, using predominantly IVP bovine embryos, nearly 15 physiological functions and expression of 60 to 70 genes have been studied (Niemann and Wrenzycki, 2000). These studies, although limited to genes of known function and sequence, demonstrate that gene expression does differ among in vivo and IVP embryos. Unfortunately, the genomic database shows about 5,800 bovine entries, but the complete sequence is known for not more than 500 of these (Kappes, 1999). Therefore, until more bovine genes are known, methods like differential display RT-PCR or restriction fragment differential display RT-PCR offer the opportunity to study a wide range of potential genes expressed during development. Embryos produced in vitro, including nuclear transfer, and derived in vivo show unique developmental competence; therefore application of the improved restriction fragment differential display to examine embryonic mRNA can lead to further discoveries in aberrant gene expression and potentially new genes involved in early bovine embryo development. With the progress in identification of new expressed sequence tags (**EST**) and genes in cattle, it will be possible to apply DNA microarray technology to investigate gene expression. However, limited amount of embryos and thus small quantity of extracted RNA could prevent successful application of this method. Not only identification of new genes, but also elucidation of their role and function must be pursued. For that purpose, the use of double-stranded (ds) RNA for sequence-specific gene silencing may be the most reliable method (Fire, 1999). The successful application of this technique to specifically inhibit gene function in mouse embryos has already been demonstrated (Wianny and Zernicka-Goetz, 2000).

Early Gene Expression in Other Mammals

In mouse embryos, changes in nucleocytoplasmatic composition caused by micromanipulation and short-term culture can influence the expression of endogenous genes (Reik et al., 1993). If there are any differences in gene expression caused by in vivo or in vitro conditions, they may have profound effects on subsequent developmental competence of embryos. Especially, disruption in the expression of imprinted genes can lead to perturbations in embryo and fetal development (Moore and Reik, 1996). The prominent example of imprinted genes in-

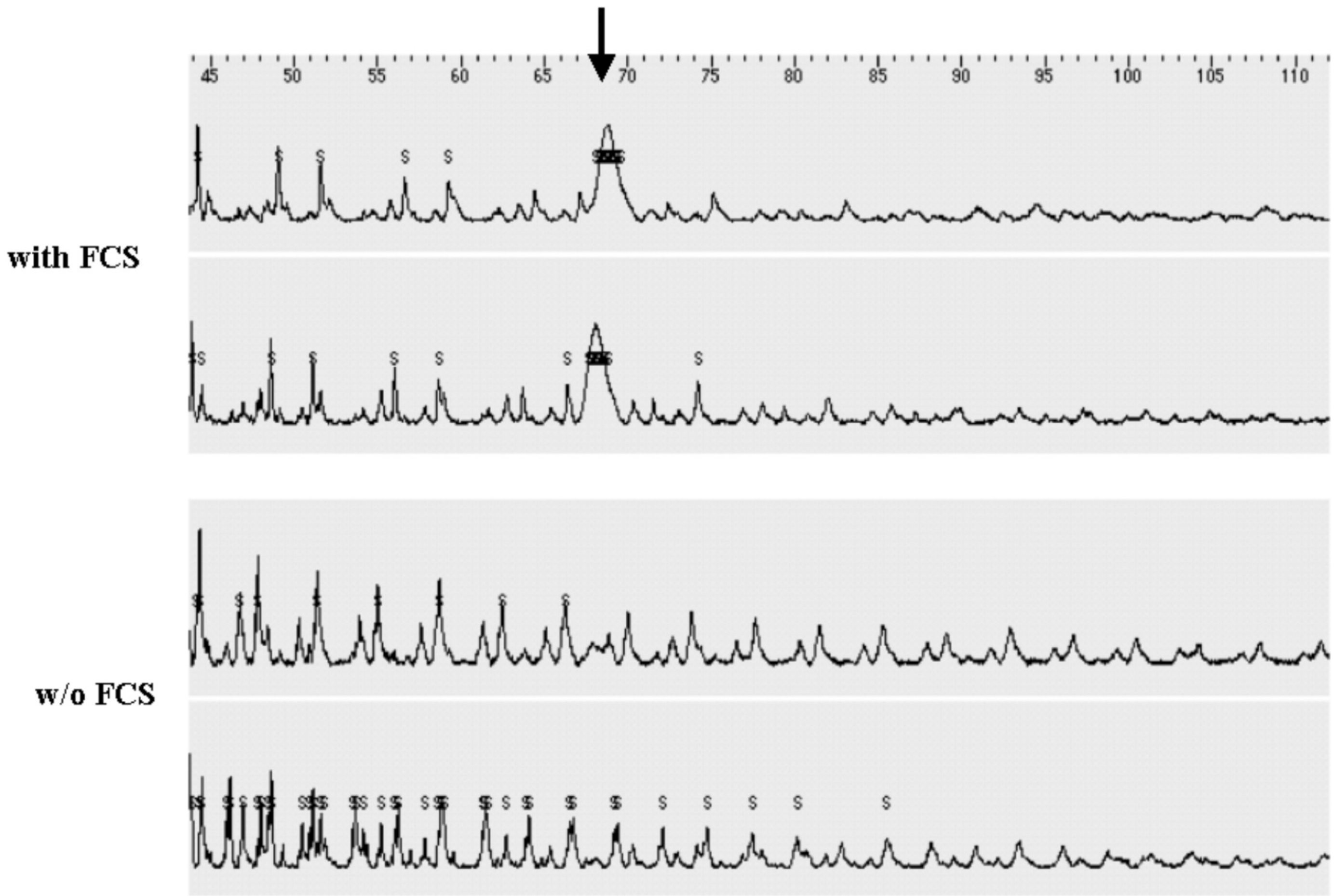

Figure 1. Differential display analysis of PCR products amplified from cDNA template derived by restriction fragment RT-PCR from bovine oocytes matured in vitro in the presence of fetal calf serum (FCS) (two upper panels) and without FCS (two bottom panels). The PCR was performed using fluorescently labeled 5′-primer and products, visualized in form of peaks, were detected using Long-Read Tower system (Visible Genetics, Inc., Toronto, Canada). Size of products increases from left to right. Arrow points on peaks represent potentially differentially expressed genes.

volved in embryonic development is expression of IGF-II and its receptor. In the mouse, the maternal chromosome produces the transcript for the receptor, and the ligand is expressed from the paternal chromosome (Barlow et al., 1991; DeChiara et al., 1991). Moreover, several additional gene transcripts have been identified in the mouse oocyte that confer its developmental competence, including a large array of growth factors, growth factor receptors, interleukins, and cytokines. Therefore, like bovine embryos, early mouse embryonic gene expression is affected by the manner in which they were produced and cultured.

Bovine In Vitro Culture Conditions

The previously mentioned studies indicate that culture conditions or factors present in media used for in vitro maturation, fertilization, and culture may trigger or exacerbate gene expression abnormalities (Figure 1). The fact that a variety of culture media are used during in vitro embryo production makes it very difficult to compare embryo quality and viability produced in different laboratories. Protein free culture media that support embryo development up to the blastocyst stage have been developed; however, in most commercial laboratories, bovine embryos are still produced with a protein (BSA or FCS) supplemented media or even with co-culture systems (Thompson and Duganzich, 1996). It is evident that inclusion of undefined components in culture media (serum, BSA) or other components such as amino acids, vitamins, and antioxidants can alter embryo metabolism and, as believed, improve embryo development (Bavister 1995). It is highly probable that changes also occur at the gene expression level (Watson et al., 2000). It is not known, however, what specific genes are affected and what their significance is for embryo development. It has been shown that in vitro culture conditions are favorable for the formation of free radicals, and they actively contribute to the occurrence of the so-called “developmental block” (Johnson and Nasr-Esfahani, 1994). Recent evidence suggests that free radicals may act as an essential second messenger in several metabolic pathways and also in the transduction-signal cascade leading to the activation of a number of tran-

scription factors (e.g., heat shock proteins, nuclear transcription factor κB, and activator protein 1; (Sen and Packer 1996; Lander, 1997; Ozolins and Hales, 1997). Inclusion of various growth factors in culture medium promotes development of bovine embryos (Larson et al., 1992). It is still unclear, however, how such factors present during in vitro culture largely affect gene expression.

Gene Expression in Nuclear Transfer Embryos

Nuclear transfer adds another dimension of complexity that can alter gene expression. The donor nucleus introduced into recipient cytoplast undergoes reprogramming in order to change the pattern of gene-expression characteristic for the adult cell into embryonic expression-specific pattern. Successful embryonic and fetal development is dependent on temporally and spatially correct expression of genes. Therefore, any changes in gene expression pattern can be manifested in embryo or fetal abnormalities. It is postulated that the appropriate reprogramming events that alter gene expression patterns from those of the donor cell to that of a normal embryo are key features for successful cloning (Campbell, 1999). In fact, it has been shown using differential display PCR that the pattern of mRNA expression in fibroblasts used as nuclear donors in nuclear reconstructed bovine blastocysts was significantly modified to become embryo-specific (De Sousa et al., 1999).

Improved Animal Production using Transgenics

Cloning combined with transgenic technology has several applications and candidate genes for biomedical application such as xenotransplantation and pharmaceutical protein production (for review see Stice et al., 1998). Cloning produced six healthy transgenic calves in 1998 (Hill et al., 1999). This was a result of transferring 110 embryos into less than 60 recipient animals. Therefore, cloning has made the production of transgenic animals much more feasible than previous microinjection technologies (Stice et al., 1998). For example, instead of injecting 36,530 one-cell embryos to get 18 transgenic offspring (0.05%; Eyestone, 1999), only 110 embryos were needed to produce six cloned transgenic offspring (5%). Also, all of the offspring produced are transgenic when cloning is used, whereas only one to ten percent of the offspring are transgenic when using traditional microinjection technology. Cloning offers major time- and cost-saving advantages to researchers producing transgenic cattle. An additional benefit, particularly for animal agriculture, is the ability to make genetic changes on a particular genotype. For example, if a particular production-tested bull has great production characteristics (i.e., feed conversion) but is lacking in another area such as marbling, then once gene(s) for improved marbling are isolated and verified, they can be added to the genotype via cell transformation in culture followed by cloning. Another major advance for transgenics has been the announcement of nuclear transfer sheep that have had a gene removed, or knocked out. However, the company (PPL Therapeutics) that produced the knockout sheep has not yet released information pertaining to how these gene knockouts were performed. This will likely be forthcoming in a patent application and later publication.

In many ways, methods of producing transgenic cattle are ahead of the animal agriculture gene discovery aspect of transgenics. Troubling is the fact that the list of potential genes to be added or knocked out to improve animal production is rather limited (Stice et al., 1998). Of great interest to the pig and poultry industries may be the removal of the GDF-8 gene, often referred to as myostatin. This gene has been naturally mutated in cattle, giving rise to the Belgian Blue breed of cattle with its increased muscling over other breeds. In cattle, this gene is problematic in the area of reproduction because Belgian Blue cattle have a high incidence of calves requiring delivery by cesarean section. In pigs and poultry, this may be a lesser issue. Another candidate gene is leptin. It may be possible to increase feed intake through modification of the leptin gene in farm animals and combine it with a modification in growth and muscling genes (C. Baile, personal communication). The SRY and other regulators of sexual phenotype are important genes to investigate to determine whether they are modifiable to produce single sex progeny. As our knowledge of the animal genome increases, we will find other genes to add or knockout to enhance production and quality traits.

Implications

Producing large numbers of cloned cattle still requires additional research. However, we are at a point where major improvements may be possible by combining cloning and transgenic technologies. Recently, gene-knockout cloned sheep were produced. Knockout cattle and pigs are not far behind. Specific gene inserts or conditional removals can now be contemplated. Cloning of cattle and pigs will present numerous opportunities to make genetic changes and multiply superior genotypes in the next few years.

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