Gene Expression in Cloned Transgenic Cattle and Swine

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Abstract:

Cloning and transgenic animal methodologies are platform technologies that will be used to make major advances in agricultural sciences, and recent progress now make it possible to combine these two powerful technologies for cattle and pigs. 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 these new 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 abnormalities appear to be at least associated but possible caused by placental abnormalities. Current 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 pre-select 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, cloning needs for transgenics is different. Producing a limited number of cloned transgenic animals is now more efficient than traditional transgenic procedures. An additional benefit for transgenics through cloning is the ability to knock out genes.

Background:

Developmental Abnormalities In Bovine Embryos

Procedures used in nuclear transfer (cloning) and in vitro embryo production contribute to the various anomalies in embryos and offspring (Stice et al., 1996 and Walker et al., 1996). High incidence of post-transfer embryo mortality is a common feature for in vitro produced (IVP) embryos. A post-day-35 mortality rate of 20-25% is very high and disturbing when compared to the rate of 5% for in vivo derived embryos (Hasler et al., 1995). In many cases, those early embryonic 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; however, in this case they may be more severe, since all embryos died by day 55 of gestation (Stice et al., 1996).

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; Sinclair et al., 1997, Farin and Farin, 1995), 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 in vitro produced 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, cardiopulmonary abnormalities were observed (Stice et al., 1996; Hill et al., 1999).

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

The above information indicates that early embryo abuses can result in anomalies observed later in gestation. Whether or not these events are a result of altered early gene expression in these embryos is debatable. Therefore it is prudent to determine first what is the range of alter gene expression in various types of embryos (in vivo, IVP, nuclear transfers). 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 a prognostic and diagnostic markers for embryonic viability.

Bovine Embryonic Gene Expression

Differences in gene expression may occur soon after the onset of transcription of embryonic genome. Interestingly, it has been thought that transition from maternal to embryonic control in cow starts 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 2-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 be seen even immediately aft 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 junctions-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 α1 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 in vitro produced 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 of 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 60-70 different genes have been studied (Niemann and Wrenzycki, 2000). These studies demonstrate that gene expression does differ among in vivo and IVP embryos; however, they were limited to genes of known function and sequence. 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 lik differential display offer the opportunity to study a wide range of potential genes expressed during development. Using the improved restriction fragment differential display on unique developmentally competent embryos can lead to further discoveries in aberrant gene expression and potentially new genes involved in early bovine embryo development.

Early Gene Expression In Mice

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 involved in embryonic development is expression of insulin-like growth factor (IGF2) and its receptor. In the mouse, the maternal chromosome produces th transcript for the receptor, while 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 an culture may trigger or exacerbate gene expression abnormalities (see figure below). The fact that a variety of culture media are used during in vitro embryo production makes it very difficult to compare the results of embryo quality and viability produced in different laboratories. Protein free culture media, which 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 non-defined components in culture medium (serum, BSA or other components such as amino acids, vitamins or 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. Previous studies show that certain conditions during in vitro culture e.g., oxidative stress can affect gene expression (Sen and Packer, 1996). It is not known, however, what specific genes are affected and what is their significance for embryo development. Inclusion of various growth factors in culture media promotes development of bovine embryos (Larson et al., 1992). It is still unclear; however, how such interventions during in vitro culture largely affect gene expression.

Another potential culture factor, which may influence early embryo development, is an oxidative stress. 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 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 transcription factors e.g., heat shock proteins nuclear transcription factor κB, activator protein 1 (Lander, 1997; Sen and Packer 1996; Ozolins and Hales, 1997). This is a clear example of how early embryo environment affects gene expression.

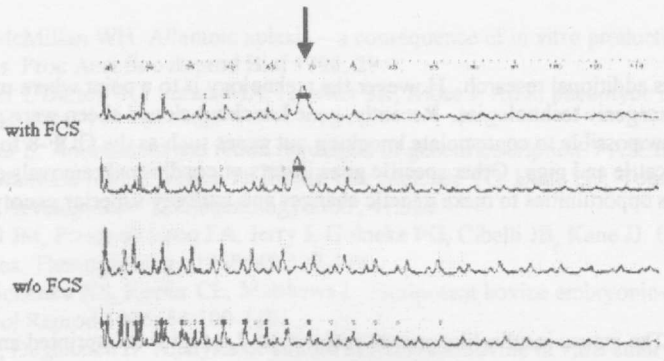

Figure 1. Fluorescent analysis of products (peaks) from single PCR reaction ("expression window" #2, two repeats). Size of products increases from left to the right. Arrow points on peak representing potentially differentially expressed genes.

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

In many ways methods of producing transgenic cattle are ahead of the gene discovery aspect of transgenics. Cloning produced 6 healthy transgenic calves in 1998. (Hill et al., 1999). This was a result of only transferring 110 embryos into less than 60 recipients animals. Therefore, cloning has made the production of transgenic animals much more easy than previous microinjection technologies (Stice et al., 1998). In other words, instead injecting 25,000 one-cell embryos to get seven transgenic offspring, only 110 embryos were needed to produce six cloned transgenic offspring. 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 provides a major time and cost saving to researchers wanting to produce transgenic cattle. An additional benefit, particularly for animal agriculture, is the ability to make genetic changes on a particular genotype. For example, particular production tested bull has great production characteristics (i.e. feed conversion) but is lacking in another area such as marbling, then after the gene(s) for that improved marbling, once isolated and verified, can be added to this particular genotype via cloning. Another major advance for transgenics has been the 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.

Troubling is the fact that the list of potential genes to be added or knockout to improve animal production is rather limited (Stice et al., 1998). Of great interest to the pig and poultry industry may the removal of the GDF-8 gene or often referred to as myostatin. This gene has been naturally mutated in cattle giving rise to the Belgium Blue breed of cattle with its increase muscling over other breeds. In cattle, this gene is problematic in the area of reproduction since the Belgium Blue has a high proportion of Cesarean section delivered calves. In pigs and poultry this is obviously a lesser issue. Another candidate gene is the leptin gene. It may be possible to increase food intake through modifying the leptin gene in farm animals and combine it with a modification in growth and muscling genes. (C. Baile, personal communication). The SRY or other upstream of downstream regulators of sexual phenotype are important genes to investigate as to determine whether they modifiable to produce single sex progeny. There are other genes and as our knowledge of the animal genome increases we will find other genes to add or knockout to enhance production and quality traits.

Conclusion

Producing large numbers of cloned cattle still requires additional research. However the technology is to 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. Therefore, it now possible to contemplate knocking out genes such as the GDF-8 for increased muscling and the SRY gene to possibly control sex ratios in cattle and pigs. Other specific gene inserts or conditional removals can be contemplated. Cloning and cattle pigs will present numerous opportunities to make genetic changes and multiply superior genotypes in the nex few years.

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