구두발표-5

Epigenetic Anomalies caused by Artificial Reproductive Technologies and Cloning by Somatic Cell Nuclear Transfer

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Introduction

Artıficial reproductive technologies (ART) have become widely accepted as useful strategıes to improve the reproductive performance and treat ınfertility cases in both domestic animals and humans. Domestic animal breeders have for long been using techniques such as artificial insemination (AI), ovarian hyperstimulation with gonadotropins to induce multiple ovulations for embryo transfer (MOET), embryo cryopreservation, and the in vitro production (IVP) of embryos by in vitro maturatıon (IVM), fertilization (IVF) and culture (IVC). More recently, techniques of animal cloning by nuclear transfer (NT) with both embryonic and somatic donor cells are being examined by the animal industry and could also become a common practicein the near future. In humans, often following the lead of farm animal practitioners, ART such as MOET, IVF, IVC and the more sophisticated procedure of intracytoplasmıc sperm injection (ICSI) have also become widely applied in fertılity clinics to help infertile couples have babies. Recent achievements by the more pioneering fertilıty clinics include the use of IVM, cytoplasmic (CT) and pronuclear (PNT) transfer to enhance the developmental competence of oocytes in the more severe cases of human infertılity.

Although the use of ART can bring obvious advantages to the dissemination of highly valuable genotypes for animal breeders and

provide opportunities for infertile couples to obtain their own offspring, such technologies have not come without problems. One of the most dramatic examples of a detrimental effect of ART in domestic animals came with the identification of the large offspring syndrome (LOS) in ruminants, which is observed in some offspring obtained by IVP and NT. In humans, IVF and ICSI have shown association with premature birth and decreased birth weight of babies and, more worryingly, some studies have indicated that children derived through such ART may show intellectual deficiencies. Notwithstanding the seriousness of the above findingsand the less noticeable high embryo mortality observed during the pre- and early post-implantation stages of development, little is known of the molecular mechanisms causing such anomalies.

Epigenetic Modifications

Recent findings seem to indicate that many of the developmental problems associated with ART may involve some kind of epigenetic anomaly. Epigenetics can be defined as heritable changes in gene function that occur without a change in the sequence of nuclear DNA. This includes the study of how environmental factors affecting a parent can result in changes in the way genes are expressed in the offspring. The object of study includes how gene regulatory information that is not expressed in DNA sequences is transmitted from one generation (of cells or organisms) to the next in addition to' the genetic information encoded in the DNA. In recent years, there has been rapid progress in understanding epigenetic mechanisms, which include differences in DNA methylation, as well as difference in chromatinstructure. Many of these studies have focused on the epigenetic effects of ART and NT and have been thoroughly reviewed in the literature [1-3]. Our purpose here is to describe a few our studies aimed at examining the epigenetic anomalies n ART- and NT-derived embryos.

DNA methylation anomalies and consequent changes to gene expression

To date, the most widely investigated epigenetic modification is DNA methylation. The methylation of DNA at cytosine residues within CpG dinucleotides is associated with transcriptional repression and is implicated in maintaining genomic stability, silencing repetitive elements and is the best understood epigenetic mark regulating genomic

imprinting. Imprinted genes are unique in that they are expressed exclusively from only one parental allele. DNA methylation is essential in facilitating this monoallelic expression by allowing one allele to be distinguished from the other. Roughly 75 imprinted genes have been identified to date in humans and mice and many play essential roles in regulating fetal growth, placental function, postnatalbehavior and several are also linked to human disease. Unfortunately, little is known to date about the regulatory mechanisms of imprinted genes in cattle, which imposes limitations to the study of epigenetic anomalies in this species. We have focused our studies on identifying the control regions of a few imprinted genes and in devising methods of analyzing the expression of these genes in a parental-specific manner.

The *Snrpn* gene

The paternally expressed bicistronic gene *Snurf-Snrpn*, hereinafter referred to as *Snrpn*, encodes a splicing factor and is among the best-studied imprinted genes to date. In both mice and humans, its imprinting has been shown to be controlled by a well characterized differentially methylated region (DMR) which acquires a maternally derived methylation imprint during oogenesis. Methylation abnormalities within the *SNRPN*DMR have been linked to the pathogenesis of Prader-Willi and Angelman syndromes, two neurodevelopmental disorders that affect children. Recently, several studies have suggested an increased incidence of Angelman and Beckwith Wiedemann (another imprinting disorder) syndromes in children conceived from IVF or ICSI technologies.

We have recently identified a CpG rich DMR within the 5' region of the bovine *Snrpn* using bisulfite sequencing [4]. *Snrpn* alleles were unmethylated in sperm, methylated in oocytes and roughly 50% methylated in somatic samples. The SnrpnDMR appeared to be hypomethylated in 2 of 6 individual 17 dpc IVF embryos examined, while alleles from 17 dpc SCNT embryos were severely hypomethylated in 4 of 5 embryos. We have also changes in the allelic expression of *Snrpn* at different stages of development. In this study, we showed that the *Snrpn*DMR methylation profiles observed in mouse and human studies are also conserved in cattle. Moreover, our work suggests that poor embryo outcome associated with *in vitro* culture and cloning can in part be explained by the aberrant methylation of imprinted genes.

The *H19* and *Igf2* genes

The *H19* and *Igf2* genes, which are close physical neighbours separated by approximately 90are believed to share a common set of enhancers, as evidenced by a coordinated expression pattern during prenatal growth and genetic dissection experiments. To explain the parent of origin-specific silencing of these genes, it is suggested that the function of the *H19* locus is that its transcriptional activity is required to imprint *Igf2* and that the conservation of its product reflects the necessity of packaging and sequestering a very abundant RNA.

To verify whether *H19* and *Igf2* genes were involved on the epigenetic anomalies of ART-derived embryos, we have identified a CpG rich DMR containing a CTCF binding site within the 5' region of the bovine *H19* gene using bisulfite sequencing [5]. H19 alleles were hypermethylated in sperm, unmethylated in oocytes and roughly 25% methylated in the liver. BothIVF and NT day-17 embryos showed hypomethylated patterns. Parental-specific transcript analysis was possible by the identification of polymorphism between *Bas taurus* and *Bos indicus* cDNA for each gene analyzed. Although in vitro-derived day-17 embryos showed a tendency of higher H19 mRNA levels, transcript amounts were not significantly different between the various groups for any of the genes analyzed. Sequence analysis showed different levels of biallelic expression of H19 in all groups. These results show that imprinted genes are aberrantly expressed in embryos produced by NT and *in vitro* culture.

The *Ascl2* gene and placental development

In mice, several genes play a role in trophoblast proliferation and differentiation, including mammalian achaete-scute complex homologue-like 2 (Ascl2; also known as Mash2) and heart and neural crest cell derivative 1 (Hand1), both basic helix-loop-helix transcription factors, which appear to have opposing activities. The Ascl2 gene stimulates cell proliferation and inhibits progression of trophoblast to their terminally differentiated giant cells form, whereas Hand1 provokes this change. Using homozygous mutant mice, it has been demonstrated that embryos with inactivated Ascl2 failed to implant and had a reduced number of spongiotrophoblast cells along with an excessive number of trophoblast giant cells. Mice with mutated Hand1 also failed to implant,

but the placenta of these fetuses lacked giant cells. Interestingly, Ascl2 is an imprinted gene in mice with the paternal allele silenced. Abundance of Ascl2 mRNA has been reported to be altered in blastocysts produced by nuclear transfer. It has also been reported the presence of fewer binucleate cells at day 60 in placenta of somatic NT bovine conceptuses compared to AI controls.

Given the morphological variation and consequent dysfunction in placentas from NT embryos, it was of considerable interest to explore the expression of factors involved in bovine trophoblast development during early stages of placental formation. We compared binucleate cell frequency and expression of genes known to be necessary for trophoblast proliferation (Ascl2), differentiation (Hand1) and function (IFN-τ and PAG-9) in bovine embryos from pregnancies from artificial insemination (AI), in vitro fertilization (IVF) and NT at days 17 and 40 of gestation arnold [6]. Significant variation was documented. Ascl2 mRNA was greatest in NT embryos compared to AI, while Hand1 was greatest in AI embryos compared to NT. IFN-τ mRNA abundance did not differ among groups. PAG-9 mRNA was undetectable in NT embryos, but greatest in AI embryos. At day 40, NT fetal cotyledons had higher Ascl2 and Hand1 than did AI tissues. Day 40 NT cotyledons had the fewest functional binucleate cells, followed by IVF and AI. Thus, genes critical for normal placental development are altered in NT bovine embryos leading to abnormal trophoblast differentiation and contributing to pregnancy loss.

Chromatin Modifications

In somatic cells, the DNA is wound about nucleosomes, which are octamers of histone proteins, and the nucleosomes are connected by linker proteins. During spermatogenesis, the histones are replaced by protamines, only to be reinstated on the male pronucleus during earlyembryogenesis. The pattern of change in oocyte histones varies from sperm and somatic cells in less understood and subtler ways. There is at least one oocyte-specific isoform of the linker histone, H1, expressed during oogenesis and early embryogenesis. It is not known to what extent these gamete-specific modifications of DNA packaging proteins occur in cloned embryos. Studies of histone dynamics in cloned animals revealed that removal of the somatic form of histone H1 from

donor chromatin has been shown to occur shortly after nuclear transfer, within 6 to 16 h, and that the temporal pattern depends on the cell cycle stage of the donor cell and of the host oocyte bordignon [7, 8]. Recent studies have further detailed the remodeling of the oocyte and somatic forms during nuclear with ES cell nuclei [9]. This first step of chromatin remodeling, involving the replacement of somatic by embryonic forms of linker histones, may be required to provide access to other, more subtle modifications to core histones. Several excellent reviews have discussed the significant advances that have been made on the epigenetic modifications of core histones and the anomalies seen in embryos derived by nuclear transfer [10, 11].

Together, it can be concluded that clear evidence is available showing that ART and particularly NT produce significant epigenetic anomalies to embryos. Since several developmental and post-natal anomalies have also been associated with these technologies, it is tempting to speculate that these and many yet undetected epigenetic anomalies are the cause of the embryonic mortality and morbidity observed in ART.

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