

# Development of fetuses from in vitro-produced and cloned bovine embryos<sup>1</sup>

C. E. Farin<sup>\*2</sup>, P. W. Farin<sup>†</sup>, and J. A. Piedrahita<sup>§</sup>

<sup>\*</sup>Department of Animal Science, <sup>†</sup>Department of Population Health and Pathobiology, and <sup>§</sup>Department of Molecular Biomedical Sciences, North Carolina State University, Raleigh 27695-7621

**ABSTRACT:** The establishment of in vitro fertilization and culture systems for mammalian embryos has facilitated the application of embryo technologies in research, industry, and clinical settings. Furthermore, the advent of cloning by nuclear transfer has significantly enhanced the potential for genetic modification of livestock. Based on studies in cattle, sheep, and mice, it has become apparent that embryos produced using these systems can differ in morphology and developmental potential compared with embryos produced in vivo. Referred to as “large offspring syndrome,” these abnormalities in the development of

fetuses, placentas, and offspring are particularly evident following transfer of cloned embryos, but they also occur in pregnancies from embryos produced using in vitro culture alone. The objective of this review is to examine the effects of in vitro production and cloning on bovine embryo and fetal development. Literature pertaining to preimplantation embryo, conceptus, and fetal development, as well as gene expression occurring at each of these three stages, is reviewed. Physiologic and genetic mechanisms that contribute to large offspring syndrome also are discussed.

Key Words: Bovine, Cloning, Fetus, In Vitro Embryo Production, Insulin-like Growth Factors

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## Introduction

Routine application of in vitro maturation (IVM), fertilization (IVF), and culture (IVC) techniques for the production of bovine embryos quickly followed the establishment of oviductal cell coculture (Eyestone and First, 1989) as a method capable of overcoming the “8- to 16-cell block” in embryo development commonly encountered in early in vitro systems. Since that time, a number of culture systems have been successfully used for in vitro production (IVP) of bovine embryos including serum/coculture-based systems, serum-free systems, and systems in which serum is introduced late in the IVC period (Farin and Farin, 1995; Hagemann et al., 1998; Hasler, 2000; Thompson, 2000; Farin et al., 2001).

Coincident with the establishment of successful IVP systems for bovine embryos, nuclear transfer was used to produce bovine embryos cloned from embryonic blastomeres (Willadsen et al., 1991; Stice and Keefer, 1993; Keefer et al., 1994; Stice et al., 1994). Subsequent developments led to the establishment of somatic cell nuclear transfer for production of mammalian embryos cloned from various cell types, including mural granulosa cells

(Wells et al., 1997), fetal fibroblasts (Campbell et al., 1996a,b; Wells et al., 2003), mammary cells (Wilmut et al., 1997), cumulus cells (Wakayama et al., 1998), and skin fibroblasts (Wakayama and Yanagimachi, 1999).

Abnormalities associated with the transfer of cloned bovine embryos were first reported by Willadsen et al. (1991) and subsequently by Keefer et al. (1994) and Wilson et al. (1995). In 1995, three reports noted abnormalities in fetuses (Farin and Farin, 1995) and calves (Behboodi et al., 1995; Sinclair et al., 1995) associated with the transfer of bovine embryos produced using IVM-IVF-IVC systems. Reported abnormalities in fetuses or calves following transfer of embryos from cloning or in vitro production (IVP) systems included lowered pregnancy rates, increased rates of abortion, production of oversized calves, musculoskeletal deformities and disproportionalities, as well as hydrallantois and other abnormalities of placental development. Collectively, these characteristics were described as “large offspring syndrome” and are now recognized to occur with high frequency following cloning of bovine embryos and with lower frequency following production of bovine embryos using IVP systems. This syndrome has also been identified in fetal and newborn lambs (Walker et al., 1996; Sinclair et al., 1999) and, more recently, in mice (Eggan et al., 2001). Interestingly, however, embryos produced by IVP or cloning systems in some species do not seem to express overt signs of large offspring syndrome. For example, cloned piglets were normally sized at birth (Archer et al., 2003); however, they exhibited increased variation in some pheno-

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<sup>2</sup>Correspondence: Box 7621, 231B Polk Hall (phone: 919-515-4022; fax: 919-515-7780; e-mail: char\_farin@ncsu.edu).

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typic traits but not others (Archer et al., 2003). The objective of this review is to examine the effects of *in vitro* production and cloning on bovine embryo and fetal development. When applicable, data from other species will also be included. Physiologic and genetic mechanisms that likely contribute to large offspring syndrome will be discussed.

### Preimplantation Development and Gene Expression

Bovine embryos produced using *in vitro* systems differ from embryos produced *in vivo* in a number of aspects. The gross morphological appearance of embryos produced *in vitro* can differ with the type of system used for embryo production. Serum-containing systems tend to result in embryos that demonstrate incomplete compaction, a darkened appearance, and less organized development of the inner cell mass compared with *in vivo* controls (Van Soom and de Kruif, 1992; Van Soom et al., 1997). In contrast, non-serum-containing systems result in the production of embryos with a more translucent appearance that undergo more complete compaction and demonstrate greater organization of the inner cell mass (Van Soom et al., 1996). In general, however, IVP systems for bovine embryos, whether they contain serum or not, are associated with more rapid development of male embryos (Avery et al., 1991, 1992; Lonergan et al., 1999), a greater accumulation of intracellular lipid (Crosier et al., 2000, 2001; Fair et al., 2001; Rizos et al., 2002a), a decrease in surface microvilli (Crosier et al., 2001; Rizos et al., 2002a), and alterations in cytoplasmic mitochondrial density (Crosier et al., 2001) when compared with embryos produced *in vivo*. For bovine embryos produced *in vivo*, development from the compact morula to blastocyst stages was associated with a doubling of the proportion of mitochondria present in the embryo cytoplasm. In contrast, bovine embryos produced *in vitro* using either serum-containing or serum-free systems failed to increase their mitochondrial density as they advanced from the compact morula to the blastocyst stages (Farin et al., 2001). Because mitochondrial function is critical for metabolic fitness and continued embryonic development, these data are consistent with the premise that *in vitro*-produced embryos are at a developmental disadvantage compared with blastocysts produced *in vivo*. Similarly, bovine embryos produced *in vitro* have a greater incidence of mixoploidy (Viuff et al., 1999; Hyttel et al., 2000) compared with embryos produced *in vivo*. In these studies, mixoploidy of the embryo was defined as the occurrence of a proportion of interphase nuclei not containing a normal diploid complement of chromosomes (Viuff et al., 2000). Embryos that exhibit polyploidy in all cells do not survive (Viuff et al., 2000). Although surviving embryos can exhibit and tolerate an increased level of mixoploidy and continue to develop (Hyttel et al., 2000), large deviations in the incidence of mixoploidy may indicate that blastocysts

produced *in vitro* are at a developmental disadvantage compared with those produced *in vivo*.

Comparisons of gene expression for bovine preimplantation embryos produced both *in vivo* and *in vitro* have demonstrated that expression of both imprinted and nonimprinted genes can be affected by *in vitro* embryo production (Wrenzycki et al., 1998; Niemann and Wrenzycki, 2000; Wrenzycki et al., 2001a; Yaseen et al., 2001; Bertolini et al., 2002a; Lazzari et al., 2002; Niemann et al., 2002; Rizos et al., 2002b; Wrenzycki et al., 2002; Rizos et al., 2003). Compared with *in vivo*-produced control embryos, levels of messenger RNA (mRNA) expression in IVP embryos can differ with stage of embryo development as well as with the base medium and supplements used for embryo culture (Wrenzycki et al., 2001a,b; Yaseen et al., 2001; Rizos et al., 2002a; Rizos et al., 2003). For example, levels of expression of a series of six genes associated with either cellular adhesion, cell metabolism, or stress responses were all altered in morula-stage bovine embryos (Wrenzycki et al., 2001a) produced using a tissue culture medium-based system compared with morulas produced *in vivo*. Comparison of this same series of genes in morulas produced using a synthetic oviductal fluid-based system demonstrated that five of these six genes were also significantly altered compared with *in vivo*-produced controls. In contrast, compared with blastocysts produced *in vivo*, levels of expression of only two of these six or one of the six genes differed in IVP blastocysts from either tissue culture medium or synthetic oviductal fluid systems, respectively (Wrenzycki et al., 2001a). Levels of expression for insulin-like growth factor (IGF)-II and the Type 1 and Type 2 IGF receptors in embryos produced *in vitro* did not differ with stage of development from the zygote to morula stages but increased significantly at the blastocyst and/or hatched blastocyst stages (Yaseen et al., 2001). When compared with blastocysts produced *in vivo*, IVP blastocysts had lower levels of expression for IGF-II, Type 1 IGF receptor, and Type 2 IGF receptor (Bertolini et al., 2002a). In contrast, other investigators have reported no differences in the level of IGF Type 2 receptor mRNA for bovine blastocysts produced *in vivo* and *in vitro* (Wrenzycki et al., 2001b). Because differences in levels of gene expression can vary within the same medium type supplemented with different commercial brands of bovine serum albumin or other type of protein (Wrenzycki et al., 2001a,b), it is not surprising that gene expression profiles for embryos at the same stage of development will differ between laboratories. Unfortunately, this situation increases the level of difficulty for successfully identifying key genes that critically influence development of IVP embryos. Analysis of expression profiles for a limited number of mRNA in bovine embryos made *in vitro* using the same culture system in two different laboratories, however, demonstrated that the levels of expression for specific genes were similarly altered between the two laboratories (Wrenzycki et al., 2001a). This observation argues for greater standardization of embryo culture conditions among laboratories in order

to more clearly identify key genes or gene groups potentially important in contributing to abnormalities associated with fetal development.

Studies of the preimplantation development of cloned embryos have focused primarily on improvement of the efficiencies of blastocyst production (Hill et al., 2000; Heyman et al., 2002), although some studies have also reported data on the quality grade of the embryos produced (Piedrahita et al., 2002; Wells et al., 2003). Data for total cell numbers in cloned blastocysts (Piedrahita et al., 2002) are consistent with those found in blastocysts produced using IVP (Farin et al., 1997; Hasler, 2000; Lane et al., 2003). In cloned bovine blastocysts, levels of expression of specific genes—including fibroblast growth factor-4, fibroblast growth factor receptor-2, interleukin-6, heat shock protein, and interferon- $\tau$ —can be influenced by donor cell type (Daniels et al., 2001) and method of donor-ooplast cell fusion (Wrenzycki et al., 2001b; Niemann et al., 2002) when compared with IVP or in vivo controls. Levels of DNA methyltransferase-1, an enzyme important in maintaining genomic methylation patterns, were elevated in IVP embryos produced either in serum-containing or serum-free media compared with in vivo controls (Wrenzycki et al., 2001b). In a second experiment, levels of DNA methyltransferase-1 were found to be elevated in cloned blastocysts when compared with IVP control blastocysts produced in serum-free medium (Wrenzycki et al., 2001b). These observations imply the occurrence of incomplete or improper reprogramming of the donor cell nucleus during the cloning procedures. In contrast, expression levels for other genes, including lactate dehydrogenase, citrate synthase, and phosphofructokinase, did not differ in cloned vs. IVP embryos (Winger et al., 2000), indicating a lack of effect of cloning procedures on reprogramming of expression.

Although the amount of data on expression of specific genes in both IVP and cloned embryos has increased significantly, a more complete understanding of the relationship between altered phenotypes and alterations in mRNA or protein expression profiles in IVP and cloned pregnancies is needed. A major difficulty in studying the effects of either specific preimplantation culture environments or particular cloning methodologies on patterns of gene expression is the lack of connection between alterations in gene expression detected at one stage of preimplantation development and subsequent effects on developmental competence during the peri- or postimplantation periods. In both mice and cattle, production of embryos using specific culture environments resulted in not only altered expression of metabolic and growth-related genes in preimplantation-stage embryos but also altered conceptus and fetal development following transfer (Khosla et al., 2001; Lazzari et al., 2002). These studies established some connection in this regard; however, they are correlative at best. The application of study designs that afford a prospective examination of causality using specifically targeted alterations in gene expression will be needed to more clearly identify the effects

of alterations of gene expression at one stage of embryo development on subsequent effects at other developmental stages. The use of novel tools such as short interfering RNA (Caplen et al., 2001; Elbashir et al., 2001; McManus and Sharp, 2002; Shi, 2003) may aid in allowing future dissection of the effects of specific, transient changes in gene expression patterns on subsequent embryo and fetal development.

### Peri-implantation Development and Gene Expression

Compared with conceptuses from embryos produced in vivo, the total length of conceptuses from embryos produced in vitro was nearly doubled at both d 12 (Lazzari et al., 2002) and 17 (Farin et al., 1999, 2001) of gestation. In contrast, at d 16 of gestation, conceptuses from embryos produced in vitro were reported to be significantly shorter than those from embryos produced in vivo (Bertolini et al., 2002a). These contrasting observations likely reflect the survival status of embryos during critical days around the time of maternal recognition of pregnancy. The observation that the majority of IVP pregnancies are lost by d 21 of gestation (Farin and Farin, 1995) suggests that a large proportion of IVP embryos fail to adequately signal for pregnancy recognition. Thus, it is likely that on d 12 of gestation most transferred embryos are still viable. Similarly, on d 16 of gestation, embryos failing to signal pregnancy recognition are likely still present in the uterus but a proportion of these are already destined for loss. By d 17 of gestation, the elongated embryos present in the uterus probably represent those who succeeded in signaling pregnancy recognition, which in cattle occurs on d 15 to 17 of gestation (Northey and French, 1980; Roberts, 1989). This suggestion is supported by the observation that both viable and degenerated conceptuses were recovered at d 17 of gestation (Farin et al., 1999, 2001).

Reports of gene expression in peri-implantation bovine conceptuses are limited. Expression of bovine interferon-mRNA occurred between d 15 to 21 of gestation (Farin et al., 1990), with a dramatic increase in expression apparent between d 15 and 16, coincident with the initiation of maternal recognition of pregnancy. In vivo-produced conceptuses recovered at d 15 of gestation expressed both IGF-I and IGF-II mRNA (Keller and Seidel, 1996). At d 16 of gestation, the expression of mRNA for IGF-I, IGF-II, Type 1 IGF receptor, Type 2 IGF receptor and the glucose transporters Glut-1 and Glut-3 did not differ between conceptuses from embryos produced in vitro compared with those from embryos produced in vivo (Bertolini et al., 2002a). There is a paucity of data regarding physical and molecular characterizations of bovine conceptuses resulting from the transfer of cloned embryos.

### Fetal Development and Gene Expression

Development of bovine fetuses following the transfer of embryos produced either in vitro or by cloning tech-

niques can be associated with the occurrence of both obvious and subtle abnormalities, including increased calf birth weight, altered organ development, altered energy metabolism, increased perinatal mortality, hydrallantois, increased pregnancy loss, and alterations in placentome number and placental structure (Kruip and den Daas, 1997; van Wagtenonk-de Leeuw et al., 1998; Hill et al., 1999; van Wagtenonk-de Leeuw et al., 2000; Farin et al., 2001; Bertolini and Anderson, 2002; Bertolini et al., 2002b; Wells et al., 2003).

Studies on fetuses resulting from the transfer of IVP embryos demonstrated disproportionate organ development in some studies (Farin and Farin, 1995; Sinclair et al., 1999) but not in others (Sangild et al., 2000; Bertolini et al., 2002b). In addition, alterations in the histological development of fetal muscle (Maxfield et al., 1997; Crosier et al., 2002) and placental tissue (Farin et al., 2000b, 2001) have been reported for pregnancies from embryos produced in vitro. For example, the ratio of secondary-to-primary muscle fiber types was increased in bovine fetuses derived from IVP embryos (Crosier et al., 2002). In contrast, individual muscle fiber areas did not differ in fetuses from embryos produced in vitro compared with those produced in vivo. Associated with these histological changes was an increase in skeletal muscle expression of the mRNA encoding glyceraldehyde-3-phosphate dehydrogenase and a decrease in expression of mRNA for myostatin in the IVP group. Levels of IGF-I mRNA in these same tissues were also elevated for the IVP group compared with controls (our unpublished results). These altered patterns of gene expression are consistent with the observed changes in muscle fiber ratios seen in histological sections taken from these same fetuses. Secondary muscle fibers have increased glycolytic capacity compared with primary muscle fibers (Dubowitz, 1960); thus, increased expression of glyceraldehyde-3-phosphate dehydrogenase mRNA could be expected in skeletal muscle demonstrating increased secondary-to-primary fiber ratios. Similarly, myostatin is known to be a negative regulator of secondary muscle fiber development, whereas IGF-I promotes myoblast proliferation and differentiation (Bass et al., 1999). Decreased expression of myostatin mRNA and increased expression of IGF-I mRNA in muscle of fetuses from IVP embryos would be consistent with the observed changes in secondary-to-primary muscle fiber ratios observed in these animals.

Assessments of mRNA and protein expression for members of the insulin-like growth factor family have been reported for fetuses following transfer of bovine as well as ovine embryos produced in vitro. Bovine fetuses at d 70 of gestation showed increased levels of IGF-II mRNA in liver tissue but tended to have decreased levels of IGF-II mRNA in skeletal muscle (Blondin et al., 2000). In contrast, at 7 mo of gestation, no differences were found in levels of IGF-II mRNA in liver or skeletal muscle (Blondin et al., 1999). For late gestation ovine fetuses selected as being significantly larger than control fetuses (thereby designated as having "large offspring syn-

drome"), levels of IGF-II mRNA in liver, kidney, heart, or skeletal muscle did not differ from controls. In contrast, levels of expression for IGF Type 2 receptor mRNA and protein were decreased in liver and muscle tissue of the selected fetuses compared with controls (Young et al., 2001). In addition, levels of IGF-binding protein-2 were elevated in plasma of the selected fetuses compared with controls (Young et al., 2001). Associated with the reduced level of expression of Type 2 receptor mRNA was a decrease in methylation levels observed within a region of the second intron (differentially methylated region 2) of the *Igf2r* gene in 9 of the 12 large fetuses. Ovine fetuses from embryos produced in vitro that were not part of the subset of 12 heaviest fetuses did not demonstrate significant differences in IGF Type 2 receptor mRNA or protein compared with controls (Young et al., 2001).

In cattle, substantial differences exist in the developmental competence of in vivo-matured cumulus oocyte complexes compared with those matured in vitro. Proportions of in vivo-matured oocytes developing to the blastocyst stage have ranged from a low of approximately 50% (Bordignon et al., 1997; Blondin et al., 2002; Rizos et al., 2002c) to a high of 80% (Blondin et al., 2002; Rizos et al., 2002c). In contrast, typical rates of blastocyst development following in vitro maturation of oocytes in the presence of gonadotropins have ranged from a low of 15% to a high of about 40% (van de Leemput et al., 1999; Choi et al., 2001; Ward et al., 2002). Currently, few data are available specifically addressing the question of the role of in vitro oocyte maturation on embryo and fetal development following embryo transfer; however, results of two studies indicate its potential importance in influencing the subsequent development of offspring following transfer of in vitro-manipulated embryos. Holm and co-workers (Holm et al., 1996) reported that birth weights of lambs derived from ovine oocytes that had been subjected to IVM and IVF followed by culture in vivo using sheep oviducts for 6.5 d prior to transfer to surrogate females were heavier than in vivo controls. More recently, Behboodi et al. (2001) reported that birth weights for transgenic calves derived from oocytes matured in vitro were significantly heavier compared with the weights of transgenic calves from oocytes matured in vivo. Unfortunately, there is a paucity of data available from experiments designed to precisely elucidate the relative roles of in vitro maturation vs. in vitro fertilization and culture on the incidence of abnormalities in conceptuses, fetuses, and offspring resulting from these procedures.

In an attempt to understand the role of oocyte competence on the outcome of nuclear transfer, comparisons of embryo, fetal, and term development of clones generated from two populations of in vitro-matured follicles have been made (Piedrahita et al., 2002). In cattle, the ability to progress to metaphase II is gradually acquired, with only 1.4% of follicles less than 0.9 mm being able to do so compared with 48% in follicles of 2 to 6 mm in diameter. The ability of the bovine oocyte to develop to the blastocyst stage is acquired when the follicle reaches 3

mm in diameter (Blondin and Sirard, 1995). Oocytes obtained from follicles smaller than 3 mm were capable of undergoing maturation and progression to MII and fertilization, but some were blocked at either the 8-cell or the 16-cell stage of development. Additionally, there was an increase in the proportion of fertilized oocytes developing to the blastocyst stage when bovine oocytes were obtained from follicles 2 to 6 mm diameter compared with those greater than 6 mm (Lonergan et al., 1994; Hagemann et al., 1999). These observations demonstrate that the developmental competence of oocytes used for in vitro fertilization procedures is optimized when oocytes are obtained from follicles whose diameters range from approximately 3 to 6 mm.

The developmental competence of bovine cytoplasts derived from two populations of follicles—1 to 3 mm diameter and 6 to 12 mm diameter—has also been compared for their effects on nuclear transfer procedures (Piedrahita et al., 2002). Cytoplasts from the two populations were fused to mural granulosa cells and the rate of development to the blastocyst stage, nuclei numbers, and pregnancy rates at d 27 of gestation and term were determined. Following in vitro maturation oocytes from 1- to 3-mm diameter follicles had a significantly lower rate of polar body formation ( $59.6 \pm 2.8\%$ ;  $n = 1,230$ ) than oocytes from the 6- to 12-mm population ( $69.0 \pm 4.6\%$ ;  $n = 857$ ). Following enucleation of oocytes demonstrating polar body extrusion, differences were detected in the fusion rate with oocytes from 6- to 12-mm follicles fusing at higher efficiency. Unexpectedly, no differences were detected in the rate of total blastocyst formation or quality of blastocysts. For analysis of developmental competence, Grade 1 and Grade 2 embryos from both follicle populations were transferred and conceptuses collected at d 27 of gestation. No differences were detected in pregnancy rate (100 vs. 80%) or fetal recovery (48 vs. 46%) between the two follicle size groups. In addition, a significant difference in allantois length (53.2 vs. 86.0 mm;  $P < 0.002$ ) and allantois width (9.5 vs. 12.8 mm;  $P < 0.06$ ) was detected between small and large follicles, respectively. Overall, conceptuses derived from follicles 1 to 3 mm in diameter possessed smaller extra-embryonic membranes. In contrast, there were no significant differences in fetal length, fetal width, or the extent of allantoic vascularization, between conceptuses derived from different-size follicles. When the developmental competency to term was compared, no differences were observed in either pregnancy rates or survival to term. The reason why oocytes from small-diameter follicles had lower developmental competence following normal fertilization but similar competence in a nuclear transfer procedure remains unclear but is most likely related to the activation protocol used in nuclear transfer. This protocol may be capable of overcoming any activation-related deficiencies in the smaller follicles. What this experiment demonstrates, however, is that immature follicles can successfully generate a cloned animal. These observations also indicate that all of the mecha-

nisms required for successful nuclear reprogramming are present early in oocyte development.

Historically, the repeated demonstration of obvious abnormalities, such as increased incidence of oversized fetuses and calves, abnormalities in placental development, and reduced pregnancy rates, have led to the original designation of “large offspring syndrome” as a general phenotypic description (Behboodi et al., 1995; Wilson et al., 1995; Kruip and den Daas, 1997; Young et al., 1998; Farin et al., 2000a, 2001; van Wagtenonk-de Leeuw et al., 2000b). However, evaluation of the distribution pattern of body weights for bovine fetuses or calves resulting from either in vitro-produced or cloned embryos, as a population, exhibit a rightward shift in weight distribution (Wilson et al., 1995; Kruip and den Daas, 1997; Farin et al., 2001). These observations imply the possibility that even animals within the presumed normal weight range may exhibit deviations in development compared with in vivo-produced controls. The contrasting perspective, that large offspring syndrome occurs in only a proportion of offspring derived from IVP or cloned embryos (i.e., the largest-sized animals) leads to the inference that those animals outside this range are essentially normal with regard to their development. To date, no clear resolution of this difference in perspectives has been achieved. However, it is important to understand the assumptions under which an experiment is pursued as this can influence the nature of sample collection and ultimately the conclusions reached about the occurrence, mechanisms, and effects of either IVP or cloning on fetuses and calves. For example, Chavatte-Palmer et al. (2002) reported that although a segment of cloned calves demonstrated obvious abnormalities associated with large offspring syndrome consistent with the hypothesis that alterations in imprinted gene expression may be driving the occurrence of this syndrome, even those calves that seemed in the normal range for birth weight demonstrated physiological perturbations. These perturbations included alterations in temperature regulation, increased abdominal fat, elevated leptin concentrations, elevated plasma IGF-II at birth, lower plasma thyroxine levels, elevated mean cell volume, and an increased neutrophil:lymphocyte ratio at birth. These physiological changes noted in normal-weight cloned calves may or may not be associated with alterations in imprinted gene expression. Similarly, changes in myostatin and glyceraldehyde-3-phosphate dehydrogenase gene expression associated with histological alterations of muscles of IVP-derived fetuses (Crosier et al., 2002) may not be directly associated with aberrant expression of imprinted genes. Therefore, on one hand, it seems obvious that perturbation of genomic imprinting is a likely cause of large offspring syndrome; on the other hand, some observations are not necessarily consistent with this hypothesis. It is possible that these other observations are physiological consequences that occur secondarily to changes in imprinted gene expression during gestation. Alternatively, they may represent additional physiological mechanisms that are perturbed as a result

of in vitro manipulations apart from changes in imprinting status. Furthermore, there may be subtle alterations of phenotypes in cloned animals that do not fall within the large offspring syndrome yet still represent epigenetic dysregulation. Using swine cloning as a model, an increase in the variability of some phenotypes of apparently normal cloned pigs has been demonstrated (Archer et al., 2003). When the methylation status of several regions of the genome were analyzed, minor differences in methylation patterns between clones and controls were observed. Although it is not possible to link the clonal variation to the epigenetic effects, it is clear that the effects of the cloning process can range from severe to very subtle. In order to resolve this issue, a more complete comparative characterization of mRNA, protein, metabolic, and epigenetic changes throughout gestation in IVP, cloned, and in vivo pregnancies is needed.

### Potential Mechanisms Underlying Large Offspring Syndrome

Currently, the leading mechanism proposed to explain the occurrence of large offspring syndrome is the alteration of epigenetic patterns associated with preimplantation embryo chromatin resulting in altered expression of imprinted genes that are important for the regulation of fetal and placental growth and development (Reik et al., 2001; Reik and Walter, 2001; Li, 2002; Reik et al., 2003b). These alterations are proposed to be induced during critical periods of preimplantation development as a result of inadequate in vitro culture conditions in the case of in vitro production systems, and/or the result of errors associated with improper genetic reprogramming of the donor cell nucleus during cloning procedures (Dean et al., 2001; Reik and Walter, 2001; Reik et al., 2003b). Epigenetic marks or patterns are defined as attributes of chromatin that can influence the expression of genes, without altering the primary DNA sequence. Epigenetic patterns can be established as a result of DNA methylation, histone modification (including methylation, deacetylation, phosphorylation), and antisense transcript formation (Reik and Walter, 2001; Li, 2002). Imprinted genes are those genes whose expression is determined by either the maternal or paternal inheritance of a specific allele (Surani, 1998). Two of the first mammalian genes to be identified as imprinted were *Igf2* and *Igf2r*, which encode for insulin-like growth factor-II and its receptor, the IGF Type 2 receptor, respectively. Whereas IGF-II mRNA is expressed only from the paternally inherited allele, mRNA for the IGF Type 2 receptor is expressed only from the reciprocal maternally inherited allele. To date, a total of 73 imprinted genes have been identified in the mouse (Mammalian Genetics Unit, <http://www.mgu.har.mrc.ac.uk/imprinting/imprintviewdatagenes.html>) and slightly fewer than 50 in the human (Moore, 2001). Estimates of the total number of imprinted genes present in the genome range from 100 to 300. These estimates are based on analyses of genomic regions in mice for which uniparental disomy and unipa-

rental duplication results in deleterious phenotypes, numbers of imprinted regions in human disease loci (Barlow, 1995), and analyses by a differential methylation screen (Bartolomei and Tilghman, 1997). Yet, analyses of uniparental disomy and duplication and numbers of imprinted regions in disease loci do not account for instances in which known imprinted genes are associated with regions where uniparental disomy or duplication do not result in abnormal phenotypes, or in cases in which regions of the genome without known imprinted genes account for imprinted phenotypes (Oakey and Beechey, 2002). Taken together, this information supports the suggestion that less than 50%, and more likely less than 25%, of the imprinted genes have been identified.

Expression of imprinted genes are believed to be primarily influenced by patterns of DNA methylation (Bartolomei et al., 1991; Li et al., 1993; Tremblay et al., 1995) in areas of gene sequence known as CpG islands, defined as regions of DNA >200 to 500 bp that have a high guanosine (G) + cytosine (C) content (>0.5) and a high density of CpG sequence pairs (Jones, 1999; Reik and Walter, 2001). Within many imprinted genes are regions of tandem repeat sequences associated with sequences that are differentially methylated between the parental alleles (differentially methylated regions). For example, the *Igf2* gene contains three differentially methylated regions, at least two of which seem to play a role in imprinted expression between the parental alleles (Moore, 2001; Lopes et al., 2003). Imprinted genes tend to be located in clusters along the chromosome and many interact with respect to their control regions. For example, expression of the *H19* gene is controlled reciprocally with the expression of the *Igf2* gene (Leighton et al., 1995; Leighton et al., 1996; Thorvaldsen et al., 1998; Lopes et al., 2003). Although DNA methylation is a well-recognized mode for control of imprinted expression for genes such as *IGF2* and *H19*, another related mode of control seems to occur through acetylation of histone proteins, which, in turn, can influence chromatin structure and accessibility of transcriptional machinery to specific gene sequences (Grandjean et al., 2001). Control of gene expression by DNA methylation and histone acetylation mechanisms may be either linked (Li, 2002) or independent (Grandjean et al., 2001), likely depending on the biological system and specific genes studied.

Primordial germ cells are freed of all epigenetic marks, primarily by DNA demethylation, through a process known as *erasure* (Reik and Walter, 2001; Dean et al., 2003). Reestablishment of paternal or maternal epigenetic marks occurs in spermatogenesis during meiosis I and in oogenesis during the period of oocyte growth (Howell et al., 2001; Li, 2002). Upon fertilization, a wave of demethylation occurs in both paternal and maternal DNA. Demethylation in paternal DNA involves an active process and results in rapid demethylation of the DNA (Kierszenbaum, 2002; Li, 2002; Reik et al., 2003b). In contrast, demethylation of the maternal DNA occurs by a passive mechanism resulting from the exclusion of

DNA methyltransferase-1, an enzyme responsible for maintenance of methylation patterns in replicating DNA (Bird, 2002), from the nucleus during early cleavage divisions (Reik et al., 2003b). In contrast to the majority of zygotic DNA, epigenetic marks on imprinted genes are protected throughout this demethylation process in both maternal and paternal DNA. Embryonic methylation patterns are reestablished after implantation in the mouse or during development to the blastocyst-stage in cattle (Reik and Walter, 2001; Li, 2002; Dean et al., 2003). This process of de novo remethylation is driven by the action of specific DNA methyltransferases-3a and -3b (Reik and Walter, 2001). Because these critical epigenetic modification events occur during processes that typically occur when manipulated embryos are exposed to artificial environments during in vitro procedures, these events have been proposed as strong candidate mechanisms related to occurrence of large offspring syndrome. The physiological changes associated with large offspring syndrome may, in part, result from aberrations in DNA methyltransferase-1 activity required for maintenance of proper methylation patterns of imprinted genes and/or aberrations in de novo remethylation activities of DNA methyltransferases-3a and -3b potentially affecting larger portions of the genome. In addition, the chromatin structure of sperm and oocyte DNA is quite different from that of a differentiated somatic cell. Thus, it is likely to affect both active and passive demethylation and result in a methylation pattern unlike that seen in normal fertilization. Furthermore, factors such as chromatin structure and histone modification may also play a role by influencing the availability of the genome not only to DNA methylases but also to transcription factors and RNA polymerases.

Observations that exposure to in vitro culture environments resulted in altered expression of a number of imprinted genes including *H19*, *Igf2*, and *Igf2r* (Ho et al., 1995; Doherty et al., 2000; Wrenzycki et al., 2001a; Bertolini et al., 2002a; Niemann et al., 2002) as well as X-linked genes (Wrenzycki et al., 2002) in preimplantation-stage embryos seems to support this hypothesis. In addition, alteration of expression for known imprinted genes in conceptuses and fetuses resulting from the transfer of IVP embryos has also been demonstrated (Blondin et al., 2000; Young et al., 2001; Bertolini et al., 2002a). Alteration of epigenetic patterns has been associated with aberrant expression of imprinted genes during subsequent embryonic, fetal, and placental development (Dean et al., 2001; Humpherys et al., 2001; Khosla et al., 2001; Young et al., 2001; Frank et al., 2002; Reik et al., 2003a). Changes in methylation pattern and levels have been associated with adverse preimplantation development (Shi and Haaf, 2002) and adverse pregnancy outcomes for cloned fetuses (Cezar et al., 2003), respectively. Although these observations certainly support of the role of altered expression of imprinted genes as a mechanism for large offspring syndrome, the additional observations of altered expression of nonimprinted genes in response to IVP or cloning procedures in both preim-

plantation-stage embryos (Wrenzycki et al., 1998; Niemann et al., 2002) and fetuses (Crosier et al., 2002) point to the occurrence of, perhaps, a more generalized mechanism. As noted earlier, these effects may simply be secondary to upstream effects on imprinted genes. Alternatively, they may indicate that in vitro culture and manipulations may influence methylation patterns that may be involved in the regulation of nonimprinted genes as has been suggested in association with tumor formation (Jones, 1999) and genetic disease (Tufarelli et al., 2003).

## Implications

Production of livestock embryos using in vitro systems represents a low-cost alternative to production of embryos in vivo. Furthermore, the use of cloning provides an alternative method for propagating genetically valuable animals and facilitates techniques for genome modification. Embryos produced using in vitro production and cloning systems can differ in morphology and developmental potential from those produced in vivo. Similarly, conceptuses, fetuses, and offspring resulting from in vitro-produced or cloned embryos can differ in their morphology, physiology, and expression of developmentally important genes compared with in vivo controls. Mechanisms proposed to explain how laboratory environments influence development of in vitro-produced and cloned embryos focus on the modification of epigenetic patterns that can affect gene expression without altering primary DNA sequences. Understanding these mechanisms will facilitate the refinement of IVP and cloning systems and the production of embryos comparable to in vivo.

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