Developmental problems during pregnancy after *in vitro* embryo manipulations[1]

Problemas gestacionais decorrentes das manipulações embrionárias in vitro

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Abstract

The *in vitro* production of bovine embryos by *in vitro* fertilization or nuclear transfer procedures is a valuable tool in wide routine use for both scientific and commercial purposes. However, this technology is associated with *in utero* abnormalities that may culminate in increased rates of pregnancy losses, hydrops of the fetal membranes, prolonged gestation, diminished signs of parturition, dystocia, and birth of large calves with lower postnatal survival. Understanding morphological, physiological and molecular features in IVP-derived pregnancies will be of significance for the prevention or attenuation of such abnormalities, with direct scientific and economical implications.

Keywords: animal cloning, nuclear transfer, *in vitro* embryo production.

Resumo

A produção in vitro (PIV) de embriões bovinos por fecundação in vitro ou transferência nuclear é uma valiosa ferramenta amplamente utilizada com propósitos científicos ou comerciais. Entretanto, esta tecnologia está associada a anormalidades gestacionais que podem culminar em elevadas perdas gestacionais, hidropsia das membranas fetais, prolongamento da gestação, menor preparação ao parto, distocia e o nascimento de bezerros absolutamente grandes com reduzida sobrevivência pós-parto. O entendimento das características morfológicas, fisiológicas e moleculares de gestações de embriões PIV será de significância para a prevenção ou redução de tais anormalidades, com repercussões econômicas e científicas diretas.

***Palavras-chave**: clonagem animal, transferência nuclear, produção in vitro de embriões.*

Introduction

It has been more than two decades since the birth of the first bovine calf (Virgil) from *in vitro* fertilization (IVF) procedures (Brackett *et al.*, 1982), and the first cloned mammal (sheep) by nuclear-transfer (NT) technology using blastomeres from preimplantation-stage embryos (Willadsen, 1986). These achievements were truly remarkable if one considers that, in those days, embryos were usually produced using *in vivo*-matured oocytes and, after IVF or NT, zygotes were transiently *in vivo*-cultured into the oviducts of surrogate females (sheep or rabbit) before the definitive embryo transfer. The subsequent development of complete *in vitro* production (IVP) systems (Lu *et al.,* 1988) not only facilitated the process but also paved the way to studies that resulted in tremendous technological advances and novel knowledge in many related areas. The pinnacle of such advancement in IVP technology was the birth of Dolly in July of 1996 by transfer of a somatic-cell nucleus from an adult ewe into an enucleated oocyte (Wilmut *et al.,* 1997). Such feat represented the fall of an important biological dogma, i.e., that differentiated somatic cells could not be reprogrammed to toti- or pluripotent state that would allow development of a new individual. In the decade that followed, cloning by NT from somatic cells, or somatic cell nuclear transfer (SCNT), has been repeated using adult, fetal and/or embryonic cells in an increasing number of domestic, wildlife and aquatic species (Table 1).

The commercial implications of such technologies can be clearly seen in Brazil, where approximately 130,000 IVF-derived embryos were reported as transferred in 2005, accounting for nearly 50% of all worldwide IVP activity, and surpassing by far the total number of *in vivo*-derived bovine embryos transferred during that same period in that country (Thibier, 2006). Brazil has also been placed on the list of the still limited number of countries in the world dominating the cloning technology by SCNT, with live born cloned calves being produced at distinct Institutions, including the groups leaded by Garcia at UNESP (Jaboticabal, SP) in 2002, Visintin at FMVZ/USP (São Paulo, SP) in 2003, Rumpf at Embrapa-Cenargen (Brasília, DF) in 2003, Meirelles at FZEA/USP (Pirassununga, SP) in 2004, Cyagra Brasil (Mogi Mirim, SP) in 2006, Ohashi at UFPA (Belém, PA)

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in 2006, and, recently, by Rodrigues at FAVET/UFRGS (Porto Alegre, RS) in 2007 (Meirelles, 2007; Rodrigues, 2007; personal communications).

Table 1. Born cloned animals by somatic cell nuclear transfer since Dolly the sheep.

Species	Cell type & origin	Reference
Sheep	Adult mammary gland cells, fetal fibroblast cells, embryonic disc-derived cells	Wilmut *et al.*, 1997
Bovine	Adult oviductal and cumulus cells	Kato *et al.,* 1998
Mouse	Adult cumulus cells	Wakayama *et al.,* 1998
Goat	Fetal fibroblast cells	Baguisi *et al.,* 1999
Pig	Adult granulosa cells	Polejaeva *et al.,* 2000
Gaur	Adult dermal fibroblast cells	Lanza *et al.*, 2000
Mouflon	Adult granulosa cells	Loi *et al.,* 2001
Cat	Adult cumulus cells	Shin *et al.*, 2002
Rabbit	Adult cumulus cells	Chesné *et al.*, 2002
Zebrafish	Embryonic fibroblast cells	Lee *et al.,* 2002
Banteng	Adult dermal fibroblast cells	Public press, 2003a
White-tailed deer	Adult dermal fibroblasts	Public press, 2003b
Mule	Fetal fibroblast cells	Woods *et al.,* 2003
Horse	Adult dermal fibroblasts	Galli *et al.*, 2003
Rat	Fetal fibroblast cells	Zhou *et al.*, 2003
Wild cat	Adult dermal fibroblasts	Gómez *et al*., 2004
Dog	Adult dermal fibroblasts	Lee *et al.*, 2005
Carp/goldfish	Common carp blastodermal cells	Sun *et al.,* 2005
Ferret	Fetal fibroblast cells, adult cumulus cells	Li *et al.*, 2006b
Gray wolf	Adult dermal fibroblast cells	Kim *et al.*, 2007

Cloning and IVF technologies have been of extraordinary scientific importance as tools for research and development. Even if still relatively inefficient, several scientific and commercial applications have also been associated with cloning by SCNT, including reproductive cloning, for the conservation and propagation of economically important individuals and endangered animals, as well presented and discussed by Meirelles *et al.* (2006), and therapeutic cloning, which may eventually have a direct impact on human health. However, pre- and postnatal deviations in development became unexpected and unpredictable consequences of *in vitro* manipulations, and have become an important animal welfare issue, also limiting the transfer of such modern reproductive technologies into commercial agricultural and biotechnology practice. Such events occurring during the first days after IVF or embryo reconstruction by NT may interfere with embryonic, fetal, and placental development *in utero*, in a set of events commonly referred to as Large Offspring Syndrome (LOS). This review aims to describe some of our current understanding of the abnormal events associated with the appearance of the syndrome, in view of normal development, with discussion of morphological, physiological and molecular aspects reported to influence conceptus growth and development after *in vitro* embryo manipulations. Before discussing abnormal events on the LOS, it is of value to describe in brief normal developmental events during pregnancy, as many known deviations from normality are related to one or more of such events.

Quick Notes on Normal Growth and Development of the Bovine Conceptus

The gestation or prenatal period, from fertilization to birth, can be divided into the embryonic and fetal phases based on the pattern of growth and development of the conceptus (Ménézo e Renard, 1993; Farin *et al.*, 2006), as illustrated in Fig. 1.

The embryonic phase is a period of differentiation, and it initiates immediately after conception (1-cell stage embryo) and continues until major organogenesis is complete (Day 42 of gestation). This phase can be further subdivided into two stages: (1) preimplantation or early embryo stage, including (A) the period of early cleavage, from conception to embryo genome activation (Days 1 to 4); (B) early embryo differentiation, when initial cell differentiation, compaction, cavitation and hatching occur (Days 4 to 10-12); and (C) elongation, with trophoblast elongation, formation of the embryonic disc and initiation of the primitive fetal membranes, and maternal recognition of pregnancy (Days 10-12 to 17-18); and (2) implantation or embryo proper stage, including the period of embryo proper; organogenesis; and placentation, in which major tissues, organs, systems,

and extraembryonic membranes are formed (Days 18 to 42). The four bovine extraembryonic membranes are derived from extraembryonic tissue during the embryonic phase (Sloss e Dufty, 1980; Guillomot *et al.*, 1993; Leiser e Kaufmann, 1994; Dantzer, 1999; Schlafer *et al.*, 2000). It is important to distinguish the origins of the yolk sac from the allantois. The yolk sac is essentially a constitutive tissue, which is, by simplification, an overexpanded blastocoele that works as a 'reservoir', supporting development during early organogenesis and placentation. The allantois expands as a pouch from the primitive gut, carrying with it the vasculature necessary for the formation of the placental vascular bed (Sloss e Dufty, 1980). The expansion of the allantois within the chorionic membrane promotes the extension of the chorioallantois along both uterine horns, and increments in allantoic fluid volume intensify the apposition of the membranes against the uterine wall, facilitating attachment, which occurs during the implantation stage between Days 17 and 22 of gestation. Attachment is as a gradual and continuous process divided into three stages: precontact (~Day 17), apposition (Days 18 to 19), and adhesion, beginning on Day 22. By Day 30 of gestation, primitive cotyledons appear in regions in apposition to caruncles. By Day 33, trophoblastic cones form villi that penetrate into caruncular crypts, producing lateral branches. By Day 45, crypts and villi are readily recognizable (Sloss e Dufty, 1980; Guillomot *et al.*, 1993; Dantzer, 1999). Embryo death during the preimplantation or implantation phases is usually referred to as early or late embryonic mortality, respectively.

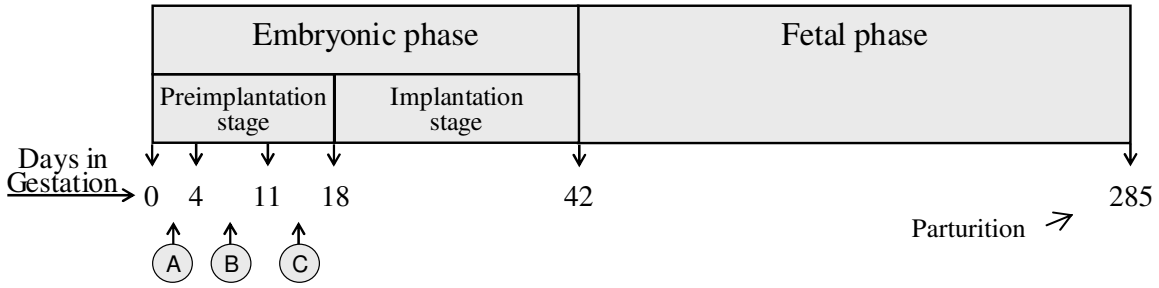

Figure 1. Divisions of the gestation period in cattle based on conceptus development. The preimplantation stage is further subdivided in (A) conception to embryo genome activation, (B) early embryo differentiation, and (C) elongation. Follow text for further details.

The period from Day 42 of gestation to term is the fetal phase, a period of rapid conceptus growth and change in conformation. Morphological changes from this period to term are modest (Sloss e Dufty, 1980). While placentomes develop and enlarge to support fetal growth, the proportion of individual organs and total fetal weight change considerably during this time (Eley *et al.*, 1978; Prior e Laster, 1979). The maximum number of placentomes can be observed by the end of the first trimester of gestation, with subsequent changes being confined to an increase in placentome size.

The Large Offspring Syndrome

The problem of abnormally large offspring was first described for calves born from blastomere NT procedures (Willadsen *et al.*, 1991; Wilson *et al.*, 1995) but was subsequently shown to occur in embryos produced by IVF (Behboodi *et al.*, 1995; Farin e Farin, 1995) and SCNT (Hill *et al.,* 1999, 2000; Renard *et al.,* 1999; Kato *et al.,* 2000) procedures. The occurrence of LOS appears to be intrinsic to the conceptus, having a worldwide distribution, with most laboratories producing IVP embryos having experienced the phenomenon. Not all offspring derived from manipulated embryos, including identical clones, are affected, demonstrating its epigenetic origin. In addition, the extent of changes can vary between individual embryos in the same culture, between NT and IVF protocols, or between laboratories (Kruip e Den Daas, 1997; Wrenzycki *et al.,* 1999, 2001; Farin *et al.,* 2001; Young *et al.,* 2001). The significance of the phenomenon is manifested by fetal and placental abnormalities that are associated with increased rates of pregnancy losses, particularly in the first 60 days of gestation (Hill *et al.,* 2000, 2001; DeSouza *et al.,* 2001; Bertolini e Anderson, 2002), and placental and fetal alterations that culminate in hydrops of the fetal membranes, late abortion, prolonged gestation, diminished signs of parturition, dystocia and birth of large calves with lower postnatal survival (Behboodi *et al.,* 1995; Wilson *et al.,* 1995; Walker *et al.,* 1996; Garry *et al.,* 1996; Hill *et al.,* 1999, 2000; Renard *et al.,* 1999; Kato *et al.,* 2000; Wells *et al.,* 2004; Batchelder *et al.*, 2005, 2007a, b; Constant *et al.*, 2006; Farin *et al.*, 2006). Because of the multitude of symptoms related to the syndrome, and due to a placenta-cause fetal-effect mechanism for the appearance of abnormal phenotypes, authors even have recently suggested the use of "Cloning Syndrome" (Wells *et al.*, 2004), "Abnormal Offspring Syndrome" (Farin *et al.*, 2006) or even "Large Placenta Syndrome" (Constant *et al.*, 2006) as more appropriate terms to describe the phenomenon.

In their comprehensive and inspiring review, and making use of scarce material, Walker *et al.* (1996)

hypothesized a series of mechanisms to explain the appearance of such abnormalities following *in vitro* embryo manipulations. Some of their suggestions have since been discounted, whereas others expanded in importance, as new pieces of evidence accumulated. Such concepts will likely be addressed by our preceding presentations on epigenetic and genetic disorders in preimplantation stage embryos and their potential role on abnormal development. This paper will focus on developmental events during late embryonic phase and the fetal phase that usually characterize the LOS.

Developmental problems during pregnancy after *in vitro* embryo manipulations

Currently, a wide range of conceptus abnormalities have been described at the morphological (macroscopic and histological), physiological (functional and metabolic) and/or molecular (epigenetic and gene expression) levels at most developmental periods for IVF- and SCNT-derived pregnancies. To facilitate research efforts towards the understanding of the phenomenon, Farin *et al.* (2006) introduced a new classification system for IVP pregnancies based on developmental outcomes of the various abnormal phenotypes, according to the degree of abnormalities: Type I, occurrence of severe abnormalities, characterized by early or late embryonic mortality (failure at distinct stages of the embryonic phase); Type II, characterized by conceptus death and abortion during the fetal phase; Type III, abnormalities compatible with a term delivery, but that inflict peri-natal death; and Type IV, moderate abnormalities compatible with a term delivery and normal or abnormal postnatal survival. Since a relatively low number of cloned embryos survive to term (1 to 5%) and approximately a third of cloned calves do not survive to weaning, most phenotypes usually fall within the first three types above. However, since more than 8% die before reaching 4 years of age (Wells *et al.,* 2004), an additional classification, a Type V phenotype, comprehending the juvenile and adult period should perhaps be included. Pieces of evidence have been suggestive of a placental-cause-fetal-effect on pre- and postnatal survival, as discussed below.

Phenotypes at the Embryonic Phase and Early Fetal Phase

Elongation phase. A limited number of reports investigating IVP concepti during the elongation period have produced some conflicting results. Bovine elongating IVP concepti have been shown to be either more developed on both Days 12 (Lazzari *et al.*, 2002) and Day 17 (Farin *et al.*, 2001) or underdeveloped on Day 16, with shorter trophoblasts and smaller embryonic discs (Bertolini *et al.*, 2002a), and on Days 12 and 14, but not on Day 10 of SCNT-derived porcine concepti (Martin *et al.*, 2007). Since medium composition used for *in vitro* embryo culture affected embryonic disc development (Fischer-Brown *et al.*, 2005), contrasting findings between studies during the elongation period may reflect differences in the IVP systems, epigenetic reprogramming or SCNT procedures. Supporting the concept of IVP conceptus underdevelopment, our results suggested the occurrence of a higher rate of early embryonic mortality. Recovery rates and embryonic disc detection for control and IVP concepti on Day 16 were 86% and 56%, and 37% and 35%, respectively (Bertolini *et al.*, 2002a). Interestingly, pregnancy rates on Days 27-30 for the same pool of IVP embryos, but for the ones allowed to develop beyond the maternal recognition of pregnancy, were 47% (51/109) and 38% (49/129) for control and IVP embryos (unpublished data), respectively. Such results suggest that a significant proportion of IVP concepti, presumably the smaller ones, were eliminated due to failure to block endometrial $PGF_{2\alpha}$ release at the time of maternal recognition of pregnancy. In addition, IVF- and SCNT-derived embryos or fetuses are smaller at early implantation stages in cattle (Bertolini *et al.*, 2002b; Chavatte-Palmer *et al.*, 2006). Consequently, it is logical to conclude that the common expected phenotype for elongating embryos involves underdevelopment of the trophoblast and/or embryonic disc. Considering the importance for the establishment of pregnancy and for the implantation period, more studies addressing deviations in development are needed during the elongation period to verify the proportion and the causes of failure for IVP embryos to signal pregnancy and promote implantation.

Implantation phase. A few reports, including ours, demonstrated the occurrence of a biphasic conceptus growth pattern in IVP pregnancies. Initially, growth restriction is observed at the end of the embryonic phase and beginning of the fetal phase (Bertolini *et al.*, 2002a; Chavatte-Palmer *et al.*, 2006). After the initial period of 'struggle', IVP fetuses that survive seem to experience a period of faster (compensatory) growth after Day 60 of gestation. This phenomenon is preceded by changes in placental development in IVF- (Bertolini *et al.,* 2002b; 2004) and SCNT-derived pregnancies (Batchelder *et al.,* 2005), restoring fetal size by the end of the first trimester of pregnancy. Such events culminate either with fetal death or with the delivery of larger IVF and SCNT calves with lower postnatal survival and morphologically altered placentas (Bertolini e Anderson, 2002; Bertolini *et al.,* 2002b; 2004, 2006; Batchelder *et al.,* 2005). Under our observations, SCNT-derived late embryos and early fetuses are even more retarded than IVF-derived counterparts (Bertolini *et al.*, 2007; unpublished data).

Early pregnancy losses. Failure in developmental events, commonly during the embryonic phase, usually leads to pregnancy losses and significant economical losses in livestock production. Late embryonic

mortality in IVP pregnancies represents the major cause for low cloning efficiency (Hill *et al.*, 2002). In cattle, pregnancy rates at 30 days for embryos derived from IVF or SCNT are usually lower than for embryos produced by superovulation or artificial insemination, but comparable to one another (30-50%). However, gestational losses between Days 30 and 60 of pregnancy are significantly higher after IVP, usually ranging from 15 to 60% for IVF, and 40 and 100% for SCNT (Wells *et al.,* 1999; Hill *et al.,* 2000; Bertolini e Anderson, 2002; Heyman *et al.,* 2002; Batchelder *et al.*, 2005).

At least two mechanistic hypotheses have been used to explain higher embryonic mortality in IVP pregnancies: (a) suboptimal IVP conditions impair development, leading to conceptus growth retardation, which in turn delays attachment and placentation, restricting further growth, and leading to death for reduced transfer capacity between the embryo and the maternal system, i.e., failure in extraembryonic membrane formation and attachment (Bertolini *et al.*, 2002a, b, 2004); and (b) expression of surface antigens from the MHC I complex in the trophoblast of SCNT-derived concepti, which may lead to rejection of the allograft (Hill *et al.*, 2002). Either possibility is not exclusive in IVP pregnancies.

The phenomenon of biphasic growth pattern is not only limited to the embryo proper or fetus, but also seems to affect membrane development, placentation and embryo survival. In previous studies, placental underdevelopment appears to precede growth restriction or even fetal demise (Hill *et al.*, 2000; Bertolini *et al.*, 2002b). Placentation and organogenesis are the major events occurring during the implantation period, and failures in either or both may be lethal. Observations by ultrasonography from pregnancies that were lost revealed that embryo development seemed to be morphologically normal in shape, but further delayed in size (unpublished data; Chavatte-Palmer *et al.*, 2006). Periods of higher lethality after SCNT occur after the blastocyst stage, with epigenetic errors affecting more severely the expression of genes associated with (Arnold *et al.*, 2006) or development of extraembryonic tissue development and placentation (Jouneau *et al.*, 2006; Wakisaka-Saito *et al.*, 2006). Then, it is unlikely that embryo growth restriction and faulty organogenesis *per se* are the primary causes in the process of losses, but possibly mere consequences of inadequate membrane attachment or placentation. In fact, poor vascularization, reduced epithelial height and low number of placentomes in Day-45 and Day-55 SCNT-derived pregnancies were associated with fetal growth retardation and high rates of early pregnancy losses in cattle and sheep (Hill *et al.*, 2000, 2002; DeSouza *et al.*, 2001). In addition, the structure of the maternal septa and fetal villi in Day-60 SCNT placentomes were less organized than controls (Hashizume *et al.*, 2002), which is not surprising in clones, considering that villous development is known to be susceptible to perturbations of genetic, epigenetic or nutritional etiology (Cross, 2006), and also that faulty epigenetic reprogramming is a common feature after cloning by SCNT (Kim *et al.*, 2005; Li *et al.*, 2005b; Arnold *et al.*, 2006; Herath *et al.*, 2006; Yang *et al.*, 2005, 2007).

Based on histological examination, Hill *et al.* (2000) suggested the existence of at least three placental phenotypes in SCNT-derived pregnancies that determine the viability of the concepti: a) underdeveloped, rudimentary placentas that cause embryonic death before or around the time for the completion of placentation (before Day 45); b) placentas with a reduced feto-maternal exchange area that would eventually lead to fetal death before Day 60 of pregnancy; and c) normal placental types, with a sporadic reduction in placentome number. Similar to the process of recognition of pregnancy, in which timing is vital, the process of placentation also has a rather narrow physiological 'window' in gestation in which development of the functional feto-maternal attachment is initiated, developed, and is completed in a rather short period (~Days 17 to 35 of pregnancy). As IVP concepti are growth-retarded immediately before and after this physiological 'window', it is logical to speculate that the concepti lost during early pregnancy were representative of one of a number of possible phenotypes that might exist during the implantation period, as suggested by Farin *et al.* (2006), which depends on the degree of abnormalities occurring during membrane and placental development.

The degree of undernourishment, hypoxia and/or toxemia by metabolite accumulation will in turn be dependent on the degree of faulty feto-maternal attachment. Such event may be more dramatically manifested after involution of the yolk sac. This explanation is consistent with timing, since losses are higher after Day 30 of gestation, when development of the chorioallantois replaces the transient yolk sac function. Due to the allantois role in attachment, placental development, and involvement in the formation of placental blood vascular system, the allantois is very important during early pregnancy, after the incipient influence of the yolk sac. In addition to reports of underdeveloped trophoblast (Hill *et al.*, 2000; Bertolini *et al.*, 2002a; Jouneau *et al.*, 2006; Wakisaka-Saito *et al.*, 2006), reports from other laboratories also indicate disturbances in yolk sac and allantois development in IVP pregnancies. Results of a recent study in which development of extraembryonic membranes in cattle was compared demonstrated significant differences in yolk sac development on Day 35 of gestation between controls (natural mating) and IVF-derived pregnancies, with the latter presenting structures that were more than 5-fold smaller than controls (Assis Neto, 2005). Interestingly, the involuting yolk sac was shown to be functionally and morphologically detectable until approximately Days 50 and 70 of pregnancy, respectively, whereas vascularization and development of cotyledons were seen only between Days 30 and 40 of pregnancy (Assis Neto, 2005). Clearly, a delay or retardation in the development of any of the two membranes may be critical for survival, when a smaller or fast-involuting yolk sac is not functionally replaced by a slow-developing

allantoic sac. In fact, Peterson e McMillan (1998a, b) and Thompson e Peterson (2000) demonstrated that the high incidence of early loss following transfer of IVP embryos was caused by malformation of the allantois, leading to placental insufficiency. Survival to Day 35 was attributed to the yolk sac, which was reported to be normal in these pregnancies. The lesions and abnormalities seen in the placental formation between these studies seem to implicate the abnormal formation of the yolk sac and/or allantois in the appearance of symptoms related to the syndrome. Altogether, underdeveloped trophoblast, precocious regression of the yolk sac and/or a failure of the allantois and vasculature to develop may delay attachment and placentation and, if threshold for survival is not attained, such events will likely be lethal.

Alternatively, Hill *et al.* (2002) have described compelling evidence demonstrating that rejection of the allograft is linked to increased late embryonic mortality in IVP pregnancies, especially for SCNT. In ruminants, suppression of MHC-I expression coincides with the intimate association between endometrial and chorioallantoic tissues during implantation (Hill *et al.*, 2002), a period that coincides with a high rate of pregnancy loss in IVP pregnancies. Clone fetuses from Days 34-63 of pregnancy were found to express MHC-I antigens that were not detected in age-matched control pregnancies (Hill *et al.*, 2002). In addition, a significant maternal lymphocytic response was also detectable in specimens expressing MHC-I peptides, with a close association with growth retardation and fetal death in earlier SCNT-derived pregnancies. It is suggested that MHC-I expression in early pregnancy is caused by faulty genome reprogramming, either by inheritance from the somatic donor cell, by persistent or continuous expression since embryo reconstruction, or by gene activation of MHC-I loci in the trophoblast as development progresses.

Phenotypes at the Fetal Phase and Perinatal Period

A little more information is available at mid- to late pregnancy, including the perinatal period. This stage is characterized by abortions, high rates of hydrops of the fetal membranes, prolonged gestation and attenuated signs of parturition, dystocia, and birth of large calves with lower postnatal survival.

Conceptus development. Fetal growth occurs slowly during early pregnancy and exponentially during later stages (Eley *et al.*, 1978; Prior e Laster, 1979; Ferrell, 1989 Reynolds *et al.*, 1990), with approximately 90% of the term fetal weight gained during the last trimester of gestation (Ferrell, 1989). However, placenta grows faster than fetus in early pregnancy, with placental weight being greater than fetal weight during the first trimester of gestation. Thereafter, fetal weight surpasses that of the placenta (Eley *et al.*, 1978; Prior e Laster, 1979; Reynolds *et al.*, 1990). However, fetal growth is normally constrained by maternal and placental factors, which is related to the control of nutrient supply to fetuses in late gestation (Ferrell, 1991a, b; Gluckman *et al.*, 1992). Disturbances in placentation may lead to changes in the pattern of fetal growth (Schlafer *et al.*, 2000). Accelerated fetal growth in IVP pregnancies is accompanied by placental enlargement, which precedes the increase in fetal weight in late IVF and SCNT pregnancies (Bertolini *et al.*, 2002b; Batchelder *et al.*, 2005; Constant *et al.*, 2006). Fetal weights are usually significantly higher than controls from mid- to late gestation (Bertolini *et al.*, 2002b; Lee *et al.*, 2004; Batchelder *et al.*, 2005; Constant *et al.*, 2006).

Our previous studies indicated IVP pregnancies to sustain larger concepti by the second trimester of pregnancy, with a two- to four-fold increase in the accumulation of of glucose and fructose in fetal plasma and associated fluids in comparison with normal pregnancies (Bertolini *et al.*, 2004). Likewise, fetal membranes of IVF and placentas of SCNT calves were noted with morphological abnormalities including two-fold increases in surface area and mass with concomitant increases in plasma fructose concentrations in IVF and cloned neonates (Bertolini *et al.*, 2004; Batchelder *et al.,* 2007a). Consequently, the larger placental mass and surface area in IVF and SCNT pregnancies suggest, at least in part, the existence of increase nutrient supply to the fetus, resulting in increased pattern of prenatal growth that may have implications to postnatal life (Bertolini *et al.,* 2004).

Rates of hydroallantois in cattle are <0.1% in normal pregnancies (1/1400 to 1/7500) and <2% in IVP pregnancies (1/55 to 1/200). However, hydrops may vary widely in SCNT pregnancies, ranging from ~0% to approximately 60% (Hasler *et al.*, 1995; Li *et al.*, 2005a; Farin *et al.*, 2006). Li *et al.* (2005a) detected significant changes in amniotic and allantoic fluid composition in clone and IVF pregnancies. In general, electrolyte homeostasis was compromised to a lesser or greater degree, suggesting problems in fetal kidney function. In contrast, our previous data did not show any significant abnormality in the fetal fluid composition on Days 90 and 180 of gestation, despite the increase in allantoic fluid volume in IVF-derived pregnancies on Day 180 (Bertolini *et al.*, 2004). Constant *et al.* (2006) recently described late gestation placentome morphometry in SCNT pregnancies complicated by hydroallantois, which may provide clues for the understanding of the pathophysiology of this abnormality during pregnancy.

Morphologically and histologically, placentas from IVF- and SCNT-derived pregnancies are significantly affected throughout gestation, with most obvious aberrations seen during mid- to late pregnancy and at term. Common observations in the third trimester of cattle and sheep IVP pregnancies and at term include placentomegaly, reduced number of placentomes, presence of giant, flattened and/or thinner placentomes, edema, enlarged umbilicus, flattened uterine epithelium, fetal connective tissue enlargement, reduced cell

density, reduced vessel dilation and density, immaturity of placental vessels, reduction of villous vascularization and vasculogenesis, hypoplasia and loss of differentiation of the trophoblastic epithelium, shed trophoblast and fetal villous hemorrhage, increased volume density of fetal pyknotic cells, among others (Bertolini *et al.*, 2002b, 2004, 2006; Lee *et al.*, 2004; Batchelder *et al.*, 2005; Miles *et al.*, 2005; Constant *et al.*, 2006; Palmieri *et al.*, 2006; Fletcher *et al.*, 2007). Authors of a recent comprehensive study have described detailed structure and microvascular architecture distinctions in bovine clone placentas (Miglino *et al.*, 2007), with the description of placentome fusions, extensive areas lacking placentation and areas of hemorrhage, and increased number of functional and accessory microcotyledons. However, the most significant ultrastructural findings referred to the presence of dilated caruncular crypts accommodating more than one primary villus with a lack of dense complexes of capillary loops and sinusoidal dilations in clone placentas. Altogether, abnormal vascularization, tissue remodeling, differentiation and maturation of placental tissue in IVP pregnancies may be the primary cause of fetal losses, abnormal fetal organ development, and faulty homeostatic control of organs and systems after birth that may compromise neonatal survival.

Parturition and mammary gland development. Other less characterized events associated with the LOS affect the end of pregnancy and include weaker or absent signals that initiate parturition, which affects the dynamics of the stages of labor, and compromised mammogenesis that seems to influence subsequent lactogenesis. A significant proportion of IVF- and SCNT-derived pregnancies fail to terminate normally, resulting in prolonged gestation. The physiological trigger for the initiation of parturition in cattle and other domestic species is the activation and maturation of the fetal adrenal cortex, which causes an increase in fetal cortisol secretion (Challis *et al.,* 2000). That will initiate a sequence of endocrine events, mainly triggered by the decrease in the progesterone/estradiol (P4/E2) ratio and the consequent release of endometrial $PGF_{2\alpha}$ (Maltier *et al.*, 1993). In sheep and most domestic animal species, parturition is stimulated by P4 withdrawal either following induction of luteolysis and/or by reduction of placental P4 production, dependent on the stage of gestation (Conley e Ford, 1987). A sharp increase in E2 synthesis is also needed for successful parturition (Challis e Thornburn, 1975). Increased E2 concentrations stimulate myometrial oxytocin receptor expression, increase in uterine contractility, prostaglandin synthesis, cervical effacement and opening, sacro-iliac relaxation and vulva distensibility (Huszar e Walsh, 1991; Jenkin e Young, 2004). Altogether, those events culminate with the maturation of the placenta and cervix, and fetal tissues and organs, delivery of the neonate, and expulsion of the fetal membranes. Such physiological events appear to be dramatically decreased in females carrying IVF- or SCNT-derived concepti. Hill e Chavatte-Palmer (2002) attempted to correlate maternal P4 profiles in the last 2 weeks of gestation of SCNT-derived pregnancies with postnatal viability of clone calves. Atypical profiles (no P4 withdrawal pre-partum) appeared to be associated with lower neonatal viability, whereas usual P4 profiles were associated with higher postnatal viability. Matsuzaki e Shiga (2002) demonstrated a lower rise in fetal cortisol levels in late SCNT-derived pregnancies, which would not be sufficient to switch the IGF system to a postnatal mode, causing difficulties during parturition. However, very little is known about the regulation of these events, how they are controlled, how they influence the initiation of parturition, and how these events are related to failures to initiate parturition and mammary gland development in IVF- and SCNT-derived pregnancies.

Postnatal development. Newborn peri- and postnatal distress and death are very common in cloned calves, frequently associated with problems in adapting to the life *ex utero*, with the frequent occurrence of breathing difficulties, reluctance to suckle, liver steatosis, abnormal kidney development, longer time to standing and sudden death (Garry *et al.*, 1996; Walker *et al.*, 1996; Hill *et al.*, 1999; Hill e Chavatte-Palmer, 2002; Chavatte-Palmer *et al.*, 2004; Wells *et al.*, 2004; Batchelder *et al.,* 2007b). Some cloned calves seem to fail to exert an effective control over the intermediate energy metabolism during the first 24 h *ex utero*, which is associated with postnatal weakness, hypoxia by pulmonary hypertension, hypo- or hyperthermia, metabolic acidosis and mortality (Garry *et al.*, 1996; Hill *et al.*, 1999; Hill e Chavatte-Palmer, 2002; Wells *et al.*, 2004; Batchelder *et al.,* 2007a). While mean plasma concentrations of many parameters including blood gases, plasma proteins, minerals, and electrolytes are similar in cloned and normal calves, clones are noted with increased variability in these shifted either higher or lower than controls suggesting subtle perturbations of homeostatic mechanisms (Batchelder *et al.*, 2007a). High neonatal mortality, clinical problems in the peripartum, and the trend for a reduced lifespan in SCNT-derived calves, with higher morbidity and mortality rates during the juvenile period, have been well described and reviewed by others (Hill *et al.,* 1999, Renard *et al.,* 1999, 2000; Kato *et al.,* 2000; Rudenko *et al.,* 2004; Wells *et al.*, 2004). Loi *et al.* (2006) have demonstrated in sheep that clones are prone to similar postnatal problems, perhaps even at greater rates than cattle. The authors have linked neonatal respiratory distress and liver and kidney degeneration primarily to placental abnormalities, attaining 100% death rate up to a month of age. Calving difficulty and the frequent need of Caesarean sections in IVF- and SCNT-derived bovine pregnancies also affect the survival and future fertility of the dam, which is translated to additional economical losses.

As more laboratories around the world continue their quest to describe phenotypes throughout development, more information will become available to better clarify the effects of *in vitro* embryo

manipulations on short- and long-term postnatal survival. In this view, the implication of the placenta as the major cause of conceptus abnormalities is not unjustified. The placenta is virtually the sole interface responsible for exchanges between fetal and maternal systems, having an important role in fetal growth by the regulation of nutrient supply and synthesis and transport of hormones, substrates, and other substances between systems. In fact, the bioavailability of certain substrates or hormones during pregnancy is important for the establishment of normal patterns of activity of physiological systems in the growing fetus, a phenomenon usually referred as metabolic programming (Mcmillen e Robinson, 2005). In this theory, changes in the pattern of substrate supply to the fetus may lead to permanent molecular and cellular modifications or even novel patterns of activities in organs and systems that may persist and affect postnatal life. Conditions such as cardiovascular diseases and diabetes originate through cardiovascular, metabolic, or endocrine adaptations that the fetus makes during undernourishment *in utero*, which change fetal programming that may persist for life. In humans, results of epidemiological studies have indicated that undernourished fetuses with low weight, thinness and short body length at birth are associated with severe effects on the children and adults during life (Barker, 1999). The concept of fetal origin to adult's illnesses (Bertram e Hanson, 2001) is based on this phenomenon, which is known as the Barker's Hypothesis (Barker, 1999). Consequently, disturbances in placenta formation due to embryo manipulations may induce changes in the metabolic reprogramming, which in turn may affect placental function. Such changes may lead to growth-related effects, altering events at peripartum, and compromising postnatal survival from birth to adulthood, as predicted by the Barker's hypothesis. Thus, the characterization of each phenotype will help us better understand how environmental or epigenetic changes during the first days in development affect pre- and postnatal life.

Molecular markers for phenotypical variations

Changes in the pattern of gene expression in preimplantation bovine embryos following *in vitro* embryo manipulations have been investigated, and it is evident that IVP conditions affect epigenetic features of the early embryo, supposedly predisposing it to faulty gene expression (Dean *et al.*, 1998; Wrenzycki *et al.*, 1999, 2001; Humpherys *et al.*, 2002; Yang *et al.*, 2007). Imprinted genes appear to be more susceptible to epigenetic alterations during IVP (Moore, 2001). Cleavage-stage embryos in sheep, cattle, and mice, or of murine stem cells seem to follow this pattern (Dean *et al.*, 1998; Humpherys *et al.*, 2001; Young *et al.* 2001; Bertolini *et al.*, 2002a). Due to the evolutionary theory of 'parental conflict' linking genomic imprinting to placentation (Haig e Graham, 1991; Moore e Haig, 1991; John e Surani, 2000), it is not surprising that abnormal epigenetic reprogramming caused by IVF or SCNT procedures, associated with *in vitro* culture conditions, may be reflected in aberrant development of the placental tissue. However, significant differences in the expression of nonimprinted genes after *in vitro* embryo manipulations have also been reported at various stages in development (Wrenzycki *et al.*, 1999, 2001; Yang *et al.*, 2007). The demonstration that certain epigenetic features (e.g., changes in DNA methylation) are common in NT-derived embryos (Bourc'his *et al.*, 2001; Kang *et al.*, 2001; Beaujean *et al.*, 2004; Yang *et al.*, 2007) only reinforce the concept that aberrant phenotypes may be potentially linked to any epigenetically modified gene. Interestingly, cloned embryos derived from less differentiated (or totipotent) cells that require little or no reprogramming (embryonic stem cells, blastomeres) develop with fewer complications after transfer to female recipients (Rideout *et al.*, 2001), despite a recent controversial report giving support to the opposite view (Sung *et al.*, 2006). Also, fertilization- and SCNT-derived ES cells were indistinguishable, based on transcriptional profiles, consistent with normal developmental potential (Brambrink *et al.*, 2006). Reprogramming of somatic cells into less differentiated cells or even into stem cells is a promising field of current intense investigation and interest (Alberio *et al.*, 2006; Hochedlinger e Jaenisch, 2006; Yang *et al.*, 2007). However, such reprogramming strategies may not be sufficient to overcome many of the biological differences that have been shown to exist between IVP and *in vivo*-derived embryos, with *in vitro* embryo production systems known to induce numerous cellular and subcellular embryonic alterations at various levels (Thompson, 1997; Holm e Callesen, 1998; Viuff *et al.*, 1999; Khurana e Niemann, 2000; Crosier *et al.*, 2001; Kang *et al.*, 2001; Kochhar *et al.*, 2001; Kuran *et al.*, 2001; Young *et al.*, 2001; Arnold *et al.*, 2006; Yang *et al.*, 2007). Collectively, such wide range variation in embryo phenotypes provides a glimpse about the level of difficulty faced to identify and establish patterns that could be used as markers for developmental abnormalities. Despite many efforts, such markers are not yet available.

Recently, many reports have stressed the importance of their results on differences in gene expression profiles between IVF-, SCNT- and/or *in vivo*-derived preimplantation stage embryos, either for single genes by real time quantitative RT-PCR or for multiple genes by the way of high throughput approaches, for individual embryos or pools of embryos (Hall *et al.*, 2005; Inoue *et al.*, 2006; Li *et al.* 2006a; Moreira *et al.*, 2006; Beyhan *et al.*, 2007; Smith *et al.*, 2007). Such results have demonstrated what everyone already knew for some time: that the embryo production system has a tremendous impact on the pattern of gene expression of embryos. Authors usually speculate, with no direct confirmation, that differences are indeed associated with abnormal development.

Interestingly, Smith *et al.* (2007) analyzed single *in vivo*-, IVP- and SCNT-derived embryos for four individual genes and concluded that culture conditions had greater effects on gene expression than NT when minimizing genetic heterogeneity. Also, Pfister-Genskow *et al.* (2005), Smith *et al.* (2005) and Somers *et al.* (2006) analyzed global gene expression in individual or in pools of control, IVP and/or SCNT embryos. Smith *et al.* (2005) also analyzed the somatic cells from which donor cells were used for NT procedures, findings that expression profiles were mostly normal between embryos, but widely different from the somatic cells, indicating that SCNT embryos were widely reprogrammed by the blastocyst stage, with less than 1% of the genes (n=50) analyzed having some differential pattern of expression between embryo types. Pfister-Genskow *et al.* (2005) and Somers *et al.* (2006) found a total of 18 and 135 differentially expressed genes, respectively, in an unpredictable manner, with no evidence for patterns for individual genes that could function as markers for abnormalities. Of importance in the study by Smith *et al.* (2005) is the fact that a very small difference was evident even between control embryos. Moreover, differences were more pronounced between IVF- and SCNT-derived embryos and controls rather than between IVF and SCNT embryos, with SCNT embryos being closer related to controls than IVF ones. Those authors suggested that developmental problems may not be due to widespread faulty reprogramming, but to abnormal gene reprogramming at the postimplantation stage.

Notwithstanding, differential patterns of expression can be more often seen at the placental level during development. Arnold *et al.* (2006) have observed expression of important genes for placental development to be altered in SCNT-derived embryos. Likewise, Humpherys *et al.* (2002) analyzed global gene expression by microarray analysis of liver and placentas of SCNT-derived newborn mice. Approximately 4% of the analyzed genes showed a significant differential pattern of expression in the placenta, with lesser differences in the liver, with most of these genes being different between the tissue types. The authors concluded that part of the abnormal gene expression may be due to the SCNT procedures, whereas others are due to the donor cell. In cattle, global expression analyses revealed a wide range gene deregulation in the liver of Day 48-56 SCNT-derived fetuses (Herath *et al.*, 2006). At term, the pattern of gene expression and protein profiles in fetuses and placental tissues have been linked to postnatal survival, with aberrant profiles characterizing deceased newborn and rather normal profiles been associated with surviving adult clones (Kim *et al.*, 2005; Li *et al.*, 2005b; Yang *et al.*, 2005). However, the identification of strong correlations between gene profiles at term and postnatal survival is of little value, currently serving more as clues for retrospective understanding of the potential etiology during prenatal development.

Under the current experimental designs and conditions, it is unlikely that studies on gene expression will reveal significant clues for the resolution of the developmental abnormalities caused by *in vitro* embryo manipulations, especially when evaluated at preimplantation stages. For that to be worthy, a change of focus is needed, perhaps for a more holistic approach to the problem, with a departure from the reductionism of the current molecular investigations. In a broad view, the evaluation of same individuals throughout development, checking molecular and physiological patterns and then following phenotypes, are essential. In addition, studies should also evaluate pregnancy losses of natural origin, to understand errors that occur in nature, including natural deviations that do not cause losses or phenotypical abnormalities.

Implications

In view of what have been discussed, we believe that many abnormalities seen after *in vitro* embryo production do occur in nature; *in vitro* embryo manipulations appear to increase their frequency of occurrence. In addition, IVP-derived animals appear to have a narrow physiological window for normality, for which trivial events for most individuals are critical or lethal. The understanding of the physiological mechanisms leading to the LOS is important for the establishment of diagnostic methods that would allow the detection and prevention of the appearance of physiological deviations during pre- and postnatal development. If such processes are identified, distinct patterns could be used as valuable markers for embryos with higher or lower developmental potential. In this view, lower pregnancy rates with higher gestational and postnatal losses represent significant economical losses for a lower prolificacy. Since an extremely high proportion of IVP-derived pregnancies are lost during early pregnancy, embryos from IVF and SCNT technologies are promising study models for embryonic mortality. However, no recognizable and trustworthy profiles of embryo abnormalities prior to transfer to female recipients that are correlated with the appearance of the LOS are yet available.

Some abnormalities observed after embryo manipulations are likely to have similar etiologies between IVF- and SCNT-derived animals, such as *in vitro* maturation and culture conditions, presence of supportive cells and animal serum during IVP procedures. It is known that such culture conditions may affect epigenetic features of cells and embryos. In turn, faulty reprogramming and chromatin remodeling may cause faulty activation/inactivation of genes, abnormal expression of imprinted and nonimprinted genes, and/or faulty initiation of transcriptional events, for which the process of SCNT is expectedly more susceptible. Pieces of evidence indicate that incomplete epigenetic reprogramming in early stages of development may initiate a chain reaction of morphological, physiological and further molecular abnormalities during pregnancy, which will

reflect in the appearance of identifiable phenotypes more readily visible by the second half of gestation or at term. Again, the early predictability of such spectrum of abnormalities in IVP pregnancies other than the conceptus demise is not yet possible.

Since placental development and function have also been associated with *in vitro* embryo manipulations, changes in placental formation, affecting its stereological structure and tissue micro-architecture, including vasculogenesis and angiogenesis, or causing further changes in placental function and metabolism, may lead to changes in the pattern of fetal development, in a placental-fetal cause-and-effect mechanism. Such mechanisms will likely result in abnormalities for the remaining of the pregnancy, even prevailing throughout the individual's life, as predicted by the Barker's Hypothesis. Consequently, morphological, physiological, metabolic and molecular studies focusing on the placenta during the periods of higher losses (embryonic phase) and faster growth and higher incidence of phenotypic deviations (second half of pregnancy and perinatal period) are essential for the understanding of the early etiology of the disorder and its relationships with placental pathologies with effects on the fetus and neonate.

Problems associated with cloning by SCNT, along with IVF, have propelled our curiosity for the understanding of causal factors, which in turn has contributed to numerous advances and generated great knowledge in many fields, such as physiology of pregnancy, placental function, epigenetic reprogramming, transgenesis, neonatology, etc. Prospective future efforts should focus on the pitfalls related to the low overall embryo production efficiency and IVP conditions; on cell differentiation, pluripotency and reprogramming; and on means to improve development after transfer by maximizing pregnancy rates, by minimizing pregnancy losses and excessive prenatal growth, and by improving the adaptation to life *ex utero*. Elucidation of causal factors behind failures is likely to continue increase our understanding of normal events in bovine reproduction and pregnancy that will assist the resolution of problems associated with the LOS and, perhaps, with those of normal occurrence in our herds. All these aspects, if better recognized, will have scientific and economical implications to agriculture and biotechnology.

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References

Alberio R, **Campbell KH**, **Johnson AD**. Reprogramming somatic cells into stem cells. *Reproduction*, v.132, n.5, p.709-720, 2006.

Arnold DR, Bordignon V, Lefebvre R, Murphy BD, Smith LC. Somatic cell nuclear transfer alters peri-implantation trophoblast differentiation in bovine embryos. Reproduction. v.132, p.279-290, 2006.

Assis Neto AC. *Desenvolvimento placentário em bovinos obtidos por gestações naturais e por fecundação in vitro*. 2005. 223f. Tese (Doutorado em Ciências) – Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia, São Paulo, 2005.

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y. Production of goats by somatic cell nuclear transfer. *Nat Biotechnol*, v.17, n.5, p.456-461, 1999.

Barker DJP. Fetal programming and public health. *In*: O'Brien PMS, Wheeler T, Barker DJP (Ed.). *Fetal programming: influences on development and disease in later life*. London: RCOG Press, 1999. p.3-11.

Batchelder CA, **Bertolini M**, **Mason JB**, **Moyer AL**, **Hoffert KA**, **Petkov SG**, **Famula TR**, **Angelos J**, **George LW**, **Anderson GB**. Perinatal physiology in cloned and normal calves: hematologic and biochemical profiles. *Cloning Stem Cells*, v.9, n.1, p.83-96, 2007b.

Batchelder CA, **Bertolini M**, **Mason JB**, **Moyer AL**, **Hoffert KA**, **Petkov SG**, **Famula TR**, **Angelos J**, **George LW**, **Anderson GB**. Perinatal physiology in cloned and normal calves: physical and clinical characteristics. *Cloning Stem Cells*, v.9, p.63-82, 2007a.

Batchelder CA, Hoffert KA, Bertolini M, Moyer AL, Mason JB, Petkov SG, Famula TR, Anderson GB. Effect of the nuclear-donor cell lineage, type, and cell donor on development of somatic cell nuclear transfer embryos in cattle. *Cloning Stem Cells*, v.7, n.4, p.238-254, 2005.

Beaujean N, **Taylor J**, **Gardner J**, **Wilmut I**, **Meehan R**, **Young L**. Effect of limited DNA methylation reprogramming in the normal sheep embryo on somatic cell nuclear transfer. *Biol Reprod,* v.71, p.185-193, 2004.

Behboodi E, Anderson GB, BonDurant RH, Cargill SL, Kreuscher BR, Medrano JF, Murray JD. Birth of large calves that developed from *in vitro*-derived bovine embryos. *Theriogenology*, v.44, p.227-232, 1995.

Bertolini M, Anderson GB. The placenta as a contributor to production of large calves. *Theriogenology*, v.57, p.181-187, 2002.

Bertolini M, Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR and Anderson GB. Growth, development and gene expression by *in vivo*- and *in vitro*-produced Day-7 and Day-16 bovine embryos. *Mol Reprod Dev*, v.63, p.318-328, 2002a.

Bertolini M, Mason JB, Beam SW, Carneiro GF, Sween ML, Moyer AL, Famula TR, Sainz RD, Anderson GB. Morphology and morphometry of *in vivo*- and *in vitro*-produced bovine concepti from early pregnancy to term and association with high birth weights. *Theriogenology*, v.58, p.973-994, 2002b.

Bertolini M, Moyer AL, Mason JB, Batchelder CA, Hoffert KA, Bertolini LR, Carneiro GF, Cargill SL, Famula TR, CC Calvert, Sainz RD, Anderson GB. Evidence of increased substrate availability to *in vitro*-derived bovine foetuses and association with accelerated conceptus growth. *Reproduction*, v.128, p.341-354, 2004.

Bertolini M, Wallace CR, Anderson GB. Expression profile and protein levels of placental products as indirect measures of placental function in *in vitro*-derived bovine pregnancies. *Reproduction*, v.131, p.163-173, 2006.

Bertram CA, Hanson MA. Animal models and programming of the metabolic syndrome. *Br Med Bull*, v.60, p.103-121, 2001.

Beyhan Z, Forsberg EJ, Eilertsen KJ, Kent-First M, First NL. Gene expression in bovine nuclear transfer embryos in relation to donor cell efficiency in producing live offspring. *Mol Reprod Dev,* v.74, p.18-27, 2007.

Bourc'his D, Le Bourhis D, Patin D, Niveleau A, Comizzoli P, Renard JP, Viegas-Pequignot E. Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr Biol*, v.11, p.1542-1546, 2001.

Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA. Normal development following *in vitro* fertilization in the cow. *Biol Reprod*, v.27, p.147-158, 1982.

Brambrink T, Hochedlinger K, Bell G, Jaenisch R. ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc Natl Acad Sci U S A, v.103, p.933-938, 2006.

Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. *Endocr Rev*, v.21, p.514-550, 2000.

Challis JR, Thorburn GD. Prenatal endocrine function and the initiation of parturition. *Br Med Bull*, v.31, p.57-61, 1975.

Chavatte-Palmer P, de Sousa N, Laigre P, Camous S, Ponter AA, Beckers JF, Heyman Y. Ultrasound fetal measurements and pregnancy associated glycoprotein secretion in early pregnancy in cattle recipients carrying somatic clones. *Theriogenology*, v.66, p.829-840, 2006.

Chavatte-Palmer P, Remy D, Cordonnier N, Richard C, Issenman H, Laigre P, Heyman Y, Mialot JP. Health status of cloned cattle at different ages. *Cloning Stem Cells*, v.6, p.94-100, 2004.

Chesné P, **Adenot PG**, **Viglietta C**, **Baratte M**, **Boulanger L**, **Renard JP**. Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat Biotechnol,* v.20, p.366-369, 2002.

Conley AJ, Ford SP. Effect of prostaglandin F2 alpha-induced luteolysis on *in vivo* and *in vitro* progesterone production by individual placentomes of cows. *J Anim Sci*, v.65, p.500-507, 1987.

Constant F, Guillomot M, Heyman Y, Vignon X, Laigre P, Servely JL, Renard JP, Chavatte-Palmer P. Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. *Biol Reprod*, v.75, p.122-130, 2006.

Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of bovine blastocysts produced in vivo or in vitro. *Biol Reprod*, v.64, p.1375-1385, 2001.

Cross JC. Placental function in development and disease. *Reprod Fertil Dev*, v.18, p.71-76, 2006.

Dantzer V. Epitheliochorial placentation. *In*: Knobil E, Neill JD (Ed.). *Encyclopedia of reproduction*. San Diego: Academic Press, 1999. p.18-28.

Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses JJ, Reik W, Feil R. Altered imprinted gene methylation and expression in completely ES cell derived mouse fetuses: association with aberrant phenotypes. *Development*, v.125, p.2273-2282, 1998.

DeSouza PA, King T, Harkness L, Young LE, Walker SK, Wilmut I. Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. *Biol Reprod*, v.65, p.23-30, 2001.

Eley RM, Thatcher WW, Bazer FW, Wilcox CJ, Becker RB, Head HH, Adkinson RW. Development of the conceptus in the bovine. *J Dairy Sci*, v.61, p.467-473, 1978.

Farin PW, Crosier AE, Farin CE. Influence of *in vitro* systems on embryo survival and fetal development in cattle. *Theriogenology*, v.55, p.151-170, 2001.

Farin PW, Farin CE. Transfer of bovine embryos produced *in vivo* or *in vitro*: survival and fetal development. *Biol Reprod*, v.52, p.676-682, 1995.

Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from *in vitro*-produced bovine embryos. *Theriogenology*, v.65, p.178-191, 2006.

Ferrell CL. Maternal and fetal influences on uterine and conceptus development in the cow: I. Growth of the tissues of the gravid uterus. *J Anim Sci*, v.69, p.1945-1953, 1991a.

Ferrell CL. Maternal and fetal influences on uterine and conceptus development in the cow: II. Blood flow and

nutrient flux. *J Anim Sci*, v.69, p.1954-1965, 1991b.
Ferrell CL. Placental regulation of fetal growth. *In*: Campion DR, Hausman GJ, Martin RJ (Ed.). *Animal growth regulation*. New York: Plenum Press, 1989. p.1-19.
Fischer-Brown AE, **Lindsey BR**, **Ireland FA**, **Northey DL**, **Monson RL**, **Clark SG**, **Wheeler MB**, **Kesler DJ**, **Lane SJ**, **Weigel KA**, **Rutledge JJ**. Embryonic disc development and subsequent viability of cattle embryos following culture in two media under two oxygen concentrations. *Reprod Fertil Dev*, v.16, p.787-793, 2005.
Fletcher CJ, Roberts CT, Hartwich KM, Walker SK, McMillen IC. Somatic cell nuclear transfer in the sheep induces placental defects that likely precede fetal demise. *Reproduction*, v.133, p.243-255, 2007.
Galli C, **Lagutina I**, **Crotti G**, **Colleoni S**, **Turini P**, **Ponderato N**, **Duchi R**, **Lazzari G**. Pregnancy: a cloned horse born to its dam twin. *Nature,* v.424, n.6949, p.635, 2003.
Garry FB, Adams R, Mccann JP, Odde KG. Postnatal characteristics of calves produced by nuclear transfer cloning. *Theriogenology*, v.45, p.141-152, 1996.
Gómez MC, **Pope CE**, **Giraldo A**, **Lyons LA**, **Harris RF**, **King AL**, **Cole A**, **Godke RA**, **Dresser BL**. Birth of african wildcat cloned kittens born from domestic cats. *Cloning Stem Cells*, v.6, p.247-258, 2004.
Gluckman PD, Morel PCH, Ambler GR, Breier BH, Blair HT, Mccutcheon SN. Elevating maternal insulin-like growth factor-I in mice and rats alters the pattern of fetal growth by removing maternal constraint. *J Endocrinol*, v.134, p.R1-R3, 1992.
Guillomot M, Fléchon JE, Leroy F. Blastocyst development and implantation. In: Thibault C, Levasseur MC, Hunter RHF. (Ed.). *Reproduction in mammals and man*. Paris: Ellipses, 1993. p.387-411.
Haig D, Graham C. Genomic imprinting and the strange case of the insulin-like growth factor II receptor. *Cell*, v.64, p.1045-1046, 1991.
Hall VJ, Ruddock NT, French AJ. Expression profiling of genes crucial for placental and preimplantation development in bovine *in vivo*, *in vitro*, and nuclear transfer blastocysts. *Mol Reprod Dev*, v.72, p.16-24, 2005.
Hashizume K, Ishiwata H, Kizaki K, Yamada O, Takahashi T, Imai K, Patel OV, Akagi S, Shimizu M, Takahashi S, Katsuma S, Shiojima S, Hirasawa A, Tsujimoto G, Todoroki J, Izaike Y. Implantation and placental development in somatic cell clone recipient cows. *Cloning Stem Cells*, v.4, p.197-209, 2002.
Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology*, v.43, p.141-152, 1995.
Herath CB, Ishiwata H, Shiojima S, Kadowaki T, Katsuma S, Ushizawa K, Imai K, Takahashi T, Hirasawa A, Takahashi S, Izaike Y, Tsujimoto G, Hashizume K. Developmental aberrations of liver gene expression in bovine fetuses derived from somatic cell nuclear transplantation. *Cloning Stem Cells*, v.8, p.79-95, 2006.
Heyman Y, Chavatte-Palmer P, LeBourhis D, Camous S, Vignon X, Renard JP. Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biol Reprod*, v.66, p.6-13, 2002.
Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin ME. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol Reprod*, v.63, p.1787-1794, 2000.
Hill JR, Chavatte-Palmer P. Pregnancy and neonatal care of cloned animals. *In*: Cibelli J, Lanza RP, Campbell KHS, West MD (Ed.). *Principles of cloning*. Amsterdam: Academic Press, 2002. p.247-266.
Hill JR, Edwards JF, Sawyer N, Blackwell C, Cibelli JB. Placental anomalies in a viable cloned calf. *Cloning*, v.3, p.83-88, 2001.
Hill JR, Roussel AJ, Cibelli JB, Edwards JF, Hooper NL, Miller NW, Thompson JA, Looney CR, Westhusin ME, Robl JM, Stice SL. Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). *Theriogenology*, v.51, p.1451-1465, 1999.
Hill JR, Schlafer DH, Fisher PJ, Davies CJ. Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is associated with a pronounced endometrial lymphocytic response. *Biol Reprod*, v.67, p.55-63, 2002.
Hochedlinger K, Jaenisch R. Nuclear reprogramming and pluripotency. *Nature*, v.441, n.7097, p.1061-1067, 2006.
Holm P, Callesen H. In vivo versus in vitro produced bovine ova: similarities and differences relevant for practical application. *Reprod Nutr Dev*, v.38, p.579-594, 1998.
Humpherys D, Eggan K, Akutsu H, Friedman A, Hochedlinger K, Yanagimachi R, Lander ES, Golub TR, Jaenisch R. Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proc Natl Acad Sci USA*, v.99, p.12889-12894, 2002.
Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout III WM, Biniszkiewwicz D, Yanagimachi R, Jaenish R. Epigenetic instability in ES cells and cloned mice. *Science*, v.293, p.95-97, 2001.
Huszar GB, Walsh MP. Relationship between myometrial and cervical functions in pregnancy and labor. *Semin Perinatol,* v.15, p.97-117, 1991.
Inoue F, Matsuda J, Ohkoshi K, Furusawa T, Takahashi S, Sasada H, Sato E, Tokunaga T. Differences in

gene expression patterns between somatic cell nuclear transfer embryos constructed with either rabbit granulosa cells or their derivatives. *Anim Reprod Sci*, v.93, p.76-87, 2006.

Jenkin G, Young IR. Mechanisms responsible for parturition; the use of experimental models. *Anim Reprod Sci*, v.82-83, p.567-581, 2004.

John RM, Surani MA. Genomic imprinting, mammalian evolution, and the mystery of egg-laying mammals. *Cell*, v.101, p.585-588, 2000.

Jouneau A, Zhou Q, Camus A, Brochard V, Maulny L, Collignon J, Renard JP. Developmental abnormalities of NT mouse embryos appear early after implantation. *Development*, v.133, p.1597-1607, 2006.

Lee KY, **Huang H**, **Ju B**, **Yang Z**, **Lin S**. Cloned zebrafish by nuclear transfer from long-term-cultured cells. *Nat Biotechnol,* v.20, p.795-799, 2002.

Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK, Han YM. Aberrant methylation of donor genome in cloned bovine embryos. *Nature Genet*, v.28, p.173-177, 2001.

Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. *Science*, v.282, n.5396, p.2095-2098, 1998.

Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J Reprod Fertil*, v.120, p.231-237, 2000.

Khurana, NK, Niemann H. Energy metabolism in preimplantation bovine embryos derived *in vitro* or *in vivo*. *Biol Reprod*, v.62, p.847-856, 2000.

Kim HR, Kang JK, Yoon JT, Seong HH, Jung JK, Lee HM, Sik Park C, Jin DI. Protein profiles of bovine placenta derived from somatic cell nuclear transfer. *Proteomics*, v.5, p.4264-4273, 2005.

Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, Hwang WS, Hossein MS, Kim JJ, Shin NS, Kang SK, Lee BC. Endangered wolves cloned from adult somatic cells. *Cloning Stem Cells,* v.9, p.130-137, 2007.

Kochhar HPS, Peippo J, King WA. Sex related embryo development. *Theriogenology*, v.55, p.3-14, 2001.

Kruip TA, Den Daas JHG. *In vitro* produced and cloned embryos: effects on pregnancy, parturition and offspring. *Theriogenology*, v.47, p.43-52, 1997.

Kuran M, Robinson JJ, Staines ME, McEvoy TG. Development and de novo protein synthetic activity of bovine embryos produced *in vitro* in different culture systems. *Theriogenology*, v.55, p.593-606, 2001.

Lanza RP, **Cibelli JB**, **Diaz F**, **Moraes CT**, **Farin PW**, **Farin CE**, **Hammer CJ**, **West MD**, **Damiani P**. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. *Cloning*, v.2, p.79-90, 2000.

Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C. Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. *Biol Reprod*, v.67, p.767-775, 2002.

Lee BC, **Kim MK**, **Jang G**, **Oh HJ**, **Yuda F**, **Kim HJ**, **Hossein MS**, **Kim JJ**, **Kang SK**, **Schatten G**, **Hwang WS**. Dogs cloned from adult somatic cells. *Nature,* v.436, n.7051, p.641, 2005.

Lee RS, Peterson AJ, Donnison MJ, Ravelich S, Ledgard AM, Li N, Oliver JE, Miller AL, Tucker FC, Breier B, Wells DN. Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary half-siblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. *Biol Reprod*, v.70, p.1-11, 2004.

Leiser R, Kaufmann P. Placental structure: in a comparative aspect. *Exp Clin Endocrinol*, v.102, p.122-134, 1994.

Li N, Wells DN, Peterson AJ, Lee RS. Perturbations in the biochemical composition of fetal fluids are apparent in surviving bovine somatic cell nuclear transfer pregnancies in the first half of gestation. *Biol Reprod,* v.73, p.139-148, 2005a.

Li S, Li Y, Du W, Zhang L, Yu S, Dai Y, Zhao C, Li N. Aberrant gene expression in organs of bovine clones that die within two days after birth. *Biol Reprod*, v.72, p.258-265, 2005b.

Li X, Amarnath D, Kato Y, Tsunoda Y. Analysis of development-related gene expression in cloned bovine blastocysts with different developmental potential. *Cloning Stem Cells*, v.8, p.41-50, 2006a.

Li Z, **Sun X**, **Chen J**, **Liu X**, **Wisely SM**, **Zhou Q**, **Renard JP**, **Leno GH**, **Engelhardt JF**. Cloned ferrets produced by somatic cell nuclear transfer. *Dev Biol,* v.293, p.439-448, 2006b.

Loi P, Clinton M, Vackova I, Fulka J Jr, Feil R, Palmieri C, Della Salda L, Ptak G. Placental abnormalities associated with post-natal mortality in sheep somatic cell clones. *Theriogenology*, v.65, p.1110-1121, 2006.

Loi P, Ptak G, Barboni B, Fulka JJr, Cappai P, Clinton, M. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nature Biotech*, v.19, p.962-964, 2001.

Lu KH, Gordon I, Gallagher M, Mcgovern H. Birth of twins after transfer of cattle embryos produced by *in vitro* techniques. *Vet Rec*, v.122, p.539-540, 1988.

Maltier JP, Legrand C, Breuiller M. Parturition. *In*: Thibault C, Levasseur MC, Hunter RHF (Ed.). *Reproduction in mammals and man*. Paris: Ellipses, 1993. p.481-501.

Martin L, Besch-Williford C, Lai L, Cheong HT, Im GS, Park KW, Murphy C, Hao Y, Ellersieck MR, Keisler DH, Schatten H, Green JA, Prather RS. Morphologic and histologic comparisons between *in vivo* and nuclear transfer derived porcine embryos. *Mol Reprod Dev*, 2007 Jan 11. [Epub ahead of print].

Matsuzaki M, Shiga K. Endocrine characteristics of cloned calves. *Cloning Stem Cells*, v.4, p.261-267, 2002.
Mcmillen CI, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev*, v.85, p.571-633, 2005.
Meirelles FV, Providelo FD, Merighe GFK, Miranda M, Traldi A, Birgel E, Valim JR, Watanabe YF. Desafios para clonagem commercial – planejando o futuro. *Acta Sci Vet*, v.34, supl.1, p.235-242, 2006.
Ménézo Y, Renard JP. The life of the egg before implantation. *In*: Thibault C, Levasseur MC, Hunter RHF (Eds.. *Reproduction in mammals and man*. Paris: Ellipses, 1993. p.349-367.
Miglino MA, Pereira FTV, Visintin JA, Garcia JM, Meirelles FV, Rumpf R, Ambrósio CE, Papa PC, Santos TC, Carvalho AF, Leiser R, Carter AM. Placentation in cloned cattle: structure and microvascular architecture. *Theriogenology*, 2007. In press.
Miles JR, Farin CE, Rodriguez KF, Alexander JE, Farin PW. Effects of embryo culture on angiogenesis and morphometry of bovine placentas during early gestation. *Biol Reprod*, v.73, p.663-671, 2005.
Moore T. Genetic conflict, genomic imprinting and establishment of the epigenotype in relation to growth. *Reproduction*, v.122, p.185-193, 2001.
Moore T, Haig D. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet*, v.7, p.45-49, 1991.
Moreira PN, Fernandez-Gonzalez R, Ramirez MA, Perez-Crespo M, Rizos D, Pintado B, Gutierrez-Adan A. Differential effects of culture and nuclear transfer on relative transcript levels of genes with key roles during preimplantation. *Zygote*, v.14, p.81-87, 2006.
Palmieri C, Loi P, Reynolds LP, Ptak G, Della Salda L. Placental abnormalities in ovine somatic cell clones at term: a light and electron microscopic investigation. *Placenta*, 2006 Oct 19. [Epub ahead of print]
Peterson AJ, McMillan WC. Allantoic aplasia – a consequence of *in vitro* production of bovine embryos and the major cause of late gestation embryo loss. Proc Aust Soc Reprod Biol, v.29, n.4, 1998a, abstr.
Peterson AJ, McMillan WC. Variation in the rate of emergence of the bovine allantois. *Proc Aust Soc Reprod Biol*, v.29, n.63, 1998b. abstract.
Pfister-Genskow M, Myers C, Childs LA, Lacson JC, Patterson T, Betthauser JM, Goueleke PJ, Koppang RW, Lange G, Fisher P, Watt SR, Forsberg EJ, Zheng Y, Leno GH, Schultz RM, Liu B, Chetia C, Yang X, Hoeschele I, Eilertsen KJ. Identification of differentially expressed genes in individual bovine preimplantation embryos produced by nuclear transfer: improper reprogramming of genes required for development. *Biol Reprod*, v.72, p.546-555, 2005.
Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dal Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KHS. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature*, v.407, p.86-89, 2000.
Prior RL, Laster DB. Development of the bovine fetus. J Anim Sci, v.48, p.1546-1553, 1979.
Public Press, 2003a**.** Advanced Cell Technology, press release, Disponible on http://www.advancedcell.com/press-release/collaborative-effort-yields-endangered-species-clone.
Public Press, 2003b. College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, press release, Disponible on http://www.cvm.tamu.edu/news/releases/2003/deer_clone.shtml.
Renard JP, Chastant S, Chesné P, Richard C, Marchal J, Cordonnier N, Chavatte P, Vignon X. Lymphoid hypoplasia and somatic cloning. *Lancet*, v.353, p.1489-1491, 1999.
Reynolds LP, Millaway DS, Kirsch JD, Infeld JE, Redmer DA. Growth and *in vitro* metabolism of placental tissues of cows from day 100 to day 250 of gestation. *J Reprod Fertil*, v.89, p.213-222, 1990.
Rideout III WR; Eggan K, Jaenish R. Nuclear cloning and epigenetic reprogramming in the genome. *Science*, v.293, p.1093-1098, 2001.
Rudenko L, Matheson JC, Adams AL, Dubbin ES, Greenlees KJ. Links Food consumption risks associated with animal clones: what should be investigated? *Cloning Stem Cells*, v.6, p.79-93, 2004.
Schlafer DH, Fisher PJ, Davies CJ. The bovine placenta before and after birth: placental development and function in health and disease. *Anim Reprod Sci*, v.60/61, p.145-160, 2000.
Shin T, **Kraemer D**, **Pryor J**, **Liu L**, **Rugila J**, **Howe L**, **Buck S**, **Murphy K**, **Lyons L**, **Westhusin M**. A cat cloned by nuclear transplantation. *Nature,* v.415, n.6874, p.859, 2002.
Sloss V, Dufty JH. (Eds). Handbook of Bovine Obstetrics. Baltimore: Williams & Wilkins Co., 1980.
Smith C, Berg D, Beaumont S, Standley NT, Wells DN, Pfeffer PL. Simultaneous gene quantitation of multiple genes in individual bovine nuclear transfer blastocysts. *Reproduction*, v.133, p.231-242, 2007.
Smith SL, Everts RE, Tian XC, Du F, Sung LY, Rodriguez-Zas SL, Jeong BS, Renard JP, Lewin A, Yang X. Global gene expression profiles reveal significant nuclear reprogramming by the blastocyst stage after cloning. *Proc Natl Acad Sci USA,* v.102, p.17582-17587, 2005.
Somers J, Smith C, Donnison M, Wells DN, Henderson H, McLeay L, Pfeffer PL. Gene expression profiling of individual bovine nuclear transfer blastocysts. *Reproduction*, v.131, p.1073-1084, 2006.
Sun YH, Chen SP, Wang YP, Hu W, Zhu ZY. Cytoplasmic impact on cross-genus cloned fish derived from transgenic common carp (*Cyprinus carpio*) nuclei and goldfish (*Carassius auratus*) enucleated eggs. *Biol*

Reprod, v.72, p.510-515, 2005.
Sung LY, Gao S, Shen H, Yu H, Song Y, Smith SL, Chang CC, Inoue K, Kuo L, Lian J, Li A, Tian XC, Tuck DP, Weissman SM, Yang X, Cheng T. Differentiated cells are more efficient than adult stem cells for cloning by somatic cell nuclear transfer. *Nat Genet*, v.38, p.1323-1328, 2006.
Thibier M. Data Retrieval Committee Annual Report: Transfer of both *in vivo* derived and *in vitro* produced embryos in cattle still on the rise and contrasted trends in other species in 2005. *Embryo Transfer Newslett*, v.24, p.12-18, 2006.
Thompson JG. Comparison between in vivo-derived and in vitro-produced pre-elongation embryos from domestic ruminants. *Reprod Fert Dev*, v.9, p.341-354, 1997.
Thompson JG, Peterson AJ. Bovine embryo culture *in vitro*: new developments and post-transfer consequences. Hum Reprod, v.15, p.59-67, 2000.
Viuff D, Rickords L, Offenberg H, Hyttel P, Avery B, Greve T, Olsaker I, Williams JL, Callesen H, Thomsen P. A high proportion of bovine blastocysts produced in vitro are mixoploid. *Biol Reprod*, v.60, p.1273-1278, 1999.
Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature*, v.394, p.369-374, 1998.
Wakisaka-Saito N, Kohda T, Inoue K, Ogonuki N, Miki H, Hikichi T, Mizutani E, Wakayama T, Kaneko-Ishino T, Ogura A, Ishino F. Chorioallantoic placenta defects in cloned mice. *Biochem Biophys Res Commun*, v.349, p.106-114, 2006.
Walker SK, Hartwich KM, Seamark RF. The production of unusually large offspring following embryo manipulation: concepts and challenges. *Theriogenology*, v.45, p.111-120, 1996.
Wells DN, Forsyth JT, McMillan V, Oback B. The health of somatic cell cloned cattle and their offspring. *Cloning Stem Cells*, v.6, p.101-110, 2004.
Wells DN, Misica PM, Tervit HR. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol Reprod*, v.60, p.996-1005, 1999.
Willadsen SM. Nuclear transplantation in sheep embryos. *Nature*, v.320, p.63-65, 1986.
Willadsen SM, Janzen RE, McAlister RJ, Shea BF, Hamilton G, McDermand D. The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. *Theriogenology*, v.35, p.161-170, 1991.
Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. *Nature*, v.385, p.810-813, 1997.
Wilson JM, Williams JD, Bondioli KR, Looney CR, Westhusin ME, McCalla DF. Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating. *Anim Reprod Sci*, v.38, p.73-83, 1995.
Woods GL, White KL, Vanderwall DK, Li GP, Aston KI, Bunch TD, Meerdo LN, Pate BJ. A mule cloned from fetal cells by nuclear transfer. *Science*, v.301, p.1063, 2003.
Wrenzycki C, Herrmann D, Carwath JW, Niemann H. Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. *Mol Reprod Dev*, v.53, p.8-18, 1999.
Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. *Biol Reprod*, v.65, p.309-317, 2001.
Yang L, Chavatte-Palmer P, Kubota C, O'neill M, Hoagland T, Renard JP, Taneja M, Yang X, Tian XC. Expression of imprinted genes is aberrant in deceased newborn cloned calves and relatively normal in surviving adult clones. *Mol Reprod Dev*, v.71, p. 431-438, 2005.
Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nat Genet*, v.39, p.295-302, 2007.
Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature Genet*, v.27, p.153-154, 2001.
Zhou Q, **Renard JP**, **Le Friec G**, **Brochard V**, **Beaujean N**, **Cherifi Y**, **Fraichard A**, **Cozzi J**. Generation of fertile cloned rats by regulating oocyte activation. *Science,* v.302, n.5648, p.1179, 2003.