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The Placenta of Bovine Clones

Pascale Chavatte-Palmer[1,2,3], Rita Sau Fong Lee[4], Sylvaine Camous[1,2], Nicolas Le Cleac'h[1,2], Hélène Jammes[1,2] & Michel Guillomot[1,2]

ABSTRACT

***Background*:** Since the first success in sheep, the production of viable cloned offspring by somatic cell nuclear transfer (SCNT) in various mammals has increased significantly. The incidence of pregnancy failure and fetal death, however, is still very high, whatever the species, and impairs the commercial development of this technology, even in the bovine species where the success rates are highest compared to other species.

***Review*:** In cattle, most gestation losses are initially due to abnormal implantation and poor placental development leading to fetal demise during the early post-implantation period (30 to 70 days of pregnancy). Thereafter, in continuing pregnancies, losses usually occur in the last third of gestation and affect about 25% of the on-going pregnancies, with very large differences according to phenotype. These are currently referred to as the Large Offspring Syndrome (LOS), Large Placenta Syndrome or Abnormal Offspring Syndrome. In all cases, the placenta appears to be central to the onset of the pathology, with placentomegaly and hydrallantois being the most common features. Clinically, transabdominal ultrasound monitoring of fetal and placental development as well as the masurement of maternal plasma concentrations of pregnancy associated glycoproteins (PAG) are recommended in order to monitor the pregnancies. Humane termination of the pregnancies by Caesarian section or slaughtering of the affected animals is recommended when the pathology onset is diagnosed more than 2 weeks prior to term. Underlying mechanisms include abnormal placental vascularization, which is present early in SCNT placental development. Enzymatic response to oxidative stress is also modified. In the first trimester, several genes expressed in the trophoblast have been found to be differentially expressed between SCNT and control conceptuses, including placental lactogen (PL), the PAG, prolactin related protein-1 (PRP-1) and Dickkopf-1(DKK-1), to name a few. All these proteins are expressed in the Binucleate cells (BNC) of the trophoblast and thus, indicate that BNC function may be affected in SCNT from very early in gestation, thereby compromising placental development. Later in pregnancy, it has been shown that transplacental exchanges are disturbed, in particular those related to glucose metabolism. Moreover, endocrine function is altered compared to controls, with decreased estrogen secretion and modifications in PAG secretion, resulting in largely elevated maternal plasma concentrations. Gene expression patterns are affected, with most prominent functional effects involving cell cycle, cell signaling pathways, molecular transport, DNA replication, recombination and repair. Most of the affected genes are downregulated. Finally, many of the pathologies reported with SCNT pregnancies resemble abnormalities reported with either mutations or deletions of imprinted genes or dysregulation of imprinted gene expression, and the expression of several imprinted genes have been shown to be abnormal in SCNT placenta.

***Conclusions*:** In conclusion, pregnancy failure after SCNT is due to multiple factors affecting, implantation, placental development, morphology, vascularization, responses to oxidative stress and the epigenetic control of gene expression. If abnormal nuclear reprogramming may induce long term effects in bovine SCNT, these effects may also be due to fetal programming due to abnormal placental function and perturbed fetal development.

Keywords: Cloning, bovine, placenta, SCNT.

CORRESPONDENCE: P. Chavatte-Palmer [pascale.chavatte@jouy.inra.fr - TEL: +33 (1) 34652558]. INRA, UMR 1198 Biologie du Développement et Reproduction, F-78350 Jouy en Josas, France. [1]INRA, UMR 1198 Biologie du Développement et Reproduction, Jouy en Josas, France. Maisons-Alfort, France. [3]PremUp Foundation, Paris, France; [4]Reproductive Technologies, AgResearch, Hamilton, New Zealand.

I. INTRODUCTION

Since the first success in sheep cloning with the production of Dolly [93], many other mammals have been cloned by somatic cell nuclear transfer (SCNT). The incidence of pregnancy failure and fetal death, however, is still very high, whatever the species. In cattle, most gestational losses are initially due to early embryonic losses during the preimplantation period [49] and thereafter to abnormal placentation associated with an overgrowth of the fetus known as the Large Offspring Syndrome (LOS) [45]. In all cases, the placenta appears to be central to the onset of clone pregnancy losses [23,50]. This article reviews what is known about the events leading to the early and late fetal losses in SCNT pregnancies and the underlying mechanisms.

II. CLINICAL AND PATHOLOGICAL OBSERVATIONS

Pregnancy losses after transfer of SCNT embryos mostly occur in the first and the third trimester of pregnancy (Figure 1) [45].

2.1 Early pregnancy losses

Following transfer of cloned bovine embryos, Day 30 pregnancy rates per recipient can approach 50%, whether or not one or several embryos have been transferred into each recipient [43,44]. After this initial pregnancy diagnosis, embryonic losses greater than 50% are common for nuclear transfer pregnancies in sheep, cattle and goats and especially for clones produced from somatic cells [21,56,89,90,93]. In contrast, only 2-4% of naturally conceived pregnancies are lost early during the first trimester (Day 30) and about 10% of *in vitro* produced (IVP) embryos have failed by Day 60. Survival of SCNT embryos to term is approximately half to 1/4 of that of *in vitro* fertilized embryos with most of these losses occurring in the first trimester [45].

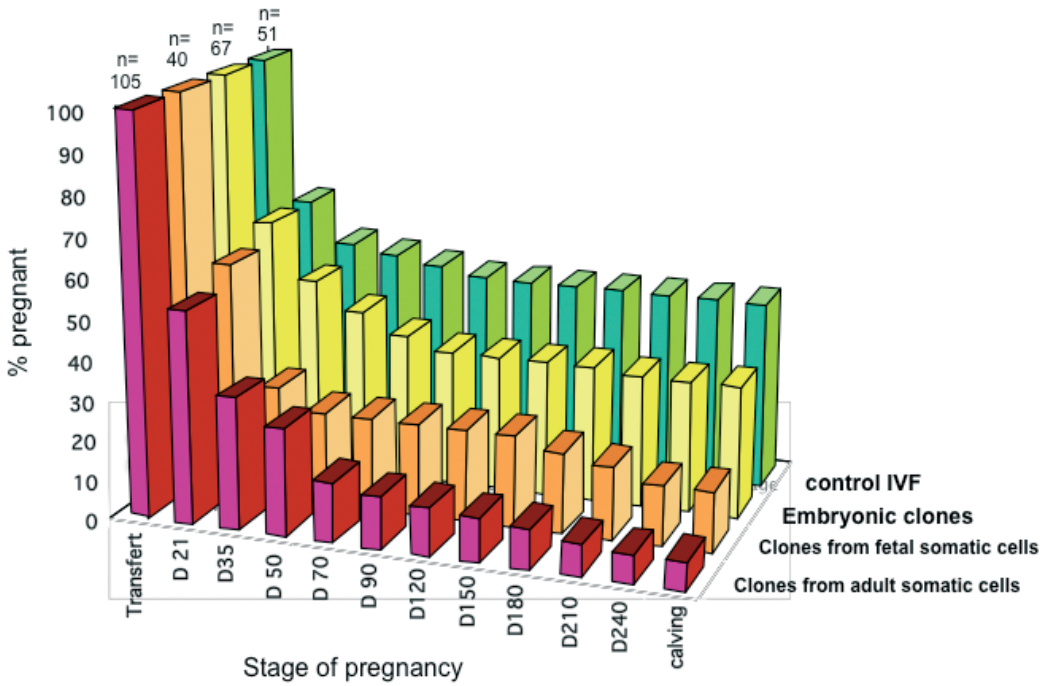

Figure 1. Schematic comparison of fetal losses throughout gestation after transfer of bovine embryos produced by IVF, NT of embryonic cells, fetal or adult fibroblasts, showing the important fetal losses in SCNT in the first and last trimester of pregnancy [45].

In naturally conceived cattle, early fetal losses may be due to abnormalities of the embryo or its placenta, alterations in maternal uterine environment or feto-maternal interactions. Possible mechanisms include chromosomal abnormalities in the embryo, hormonal changes, environmental influences, asynchronous embryo transfer and immunological rejection [92]. In SCNT pregnancies, these same factors generally still apply. The lack of, or abnormal placentome development, initially observed in bovine fetuses NT from embryonic cells [83], is the most common observation at Day 35-50. First trimester SCNT fetuses display a wide variety of placental morphologies with poor allantoic vascularization, retarded or even advanced cotyledon development [58] and formation of smaller numbers of placentomes [6,28,41,50,90]. SCNT fetuses have very variable numbers of placentomes, which suggests that the completeness of placental development varies widely in cloned animals. When placentome numbers are decreased by as much as 80%, the is a very high risk for the pregnancy to be lost before Day 90 of gestation [58]. Similar placental abnormalities were also shown to occur in first trimester bovine IVF fetuses, in relation to the use of sub-optimal embryo culture media prior to embryo transfer [32,33].

Fetuses recovered shortly after death from such pregnancies are grossly normal most of the time, and thus it appears that lack of normal placentation may be a major contributor to embryonic death, rather than fetal abnormalities *per se*. Abnormal placental development and impaired function, such as inadequate maternal-fetal contact and poor transfer of nutrients likely contribute to starvation of the developing fetus [11,17].

2.2. Late pregnancy Losses

In bovine SCNT pregnancies, fetal losses occur sporadically throughout the second and third trimesters. In many of these cases, decreased numbers of placentomes are over-compensated with increased total placental mass [16,23,66,72]. Enlarged placentomes are not only larger in area but coronal sections are much thicker. A similar reduction in placentome numbers together with an increase in placentome size has been achieved in sheep following surgical carunculectomies of the uterus prior to getting the females pregnant; however, growth compensation of the placentomes was not sufficient to allow the fetuses to grow at a normal rate [77], in contrast to what happens in clones, where fetal oversize is a common feature despite reduced placentome numbers [9,19,23].

Occasionally, microplacentomes are observed, where large numbers of non-discrete placentomes form "mats" in the uterus. This and the observation of reduced numbers of placentomes suggest that placentomes, which should only form in relation to discrete areas, the endometrial caruncles, have failed to form on many of the caruncles or have fused together or are forming indiscriminately on the endometrium (mats of microplacentomes). This may also be the result of physiological adaptations to try to increase placental transfer capacity [66].

Eventually, a high proportion of the fetal losses may be ascribed to placental-associated abnormalities, such as hydrallantois and edema of the fetal membranes and placental tissues. The enlarged placentomes and edematous membranes can be visualized by trans-abdominal or trans-rectal ultrasonography (Figure 2) or diagnosed by trans-rectal palpation.

Euthanasia of pathological cases showed that placentome enlargement sometimes seems to affect only part of the placenta with areas with normal and areas with edematous placentomes. The lesions probably spread as gestation continues. During perfusion fixation of some clone placentomes, what appears to be microthrombi have sometimes been observed. Histological examination of edematous placentomes from clones showed that although edema was present, there was little or no inflammatory process and the fetal and maternal tissue in the placentome both appeared grossly normal [23]. However, together with hydrallantois, fetal lesions, including omphalocele, ascites, cardiac enlargement, liver steatosis [18,19] are often present, together with frequent renal lesions such as hydronephrosis, some of which may be related to the observed placental abnormalities [11,18].

III. VETERINARY CARE OF THE BOVINE SCNT PREGNANCY

3.1. Detection of the early losses

Ultrasonography in farm animals has been useful to demonstrate that a high rate of fetal death in SCNT pregnancies occurs from the first to third month of gestation [17,28,48,72]. In the early stages, the fetuses that are destined to die become progressively more growth retarded for their gestational age but it is not easy to precociously predict, using ultrasound

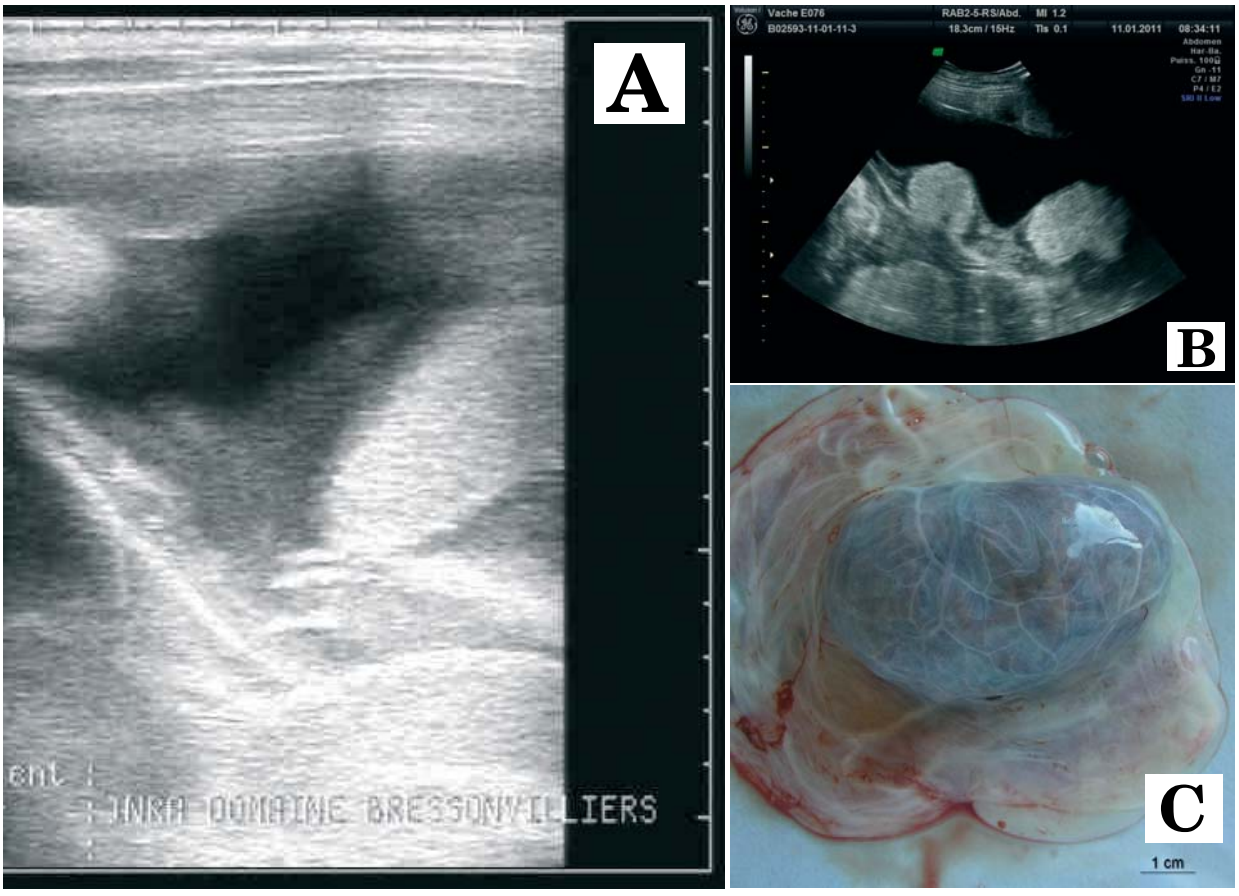

Figure 2. Transabdominal ultrasound view of an abnormal placentome in a SCNT pregnancy showing the hyperechogenic texture and the edema surrounding the placentome and cloudy fluids A) image obtained with a Vetscan®, 3.5 MHz probe, B) image obtained with a Voluson i, (GE Medical Systems) equipped with a transabdominal multifrequency probe (2.2-6.5 MHz). C) Photograph of an enlarged placentome with edema.

examination, which fetuses are ultimately non-viable [17]. A reasonable rate for monitoring SCNT pregnancies by trans-rectal scanning is every 15 days, beginning at Day 35. At 70-80 days of age, after the completion of implantation, the amniotic sacs of some cloned fetuses already appear to be enlarged and, at post-mortem examination, these fetuses had enlarged livers [47].

In the first trimester, placental development can also be evaluated by maternal levels of serum proteins such as pregnancy specific protein B (PSPB) [82], pregnancy associated glycoprotein (bPAG) [98] and pregnancy serum protein 60, a protein of 60 kDa (PSP60) [65]. Except for some biochemical differences, these proteins all belong to the large family of Pregnancy-Associated Glycoproteins or the PAGs. PSP60, secreted by the binucleate cells of the placenta, as most PAGs, is a specific marker of pregnancy in cattle and easily assayed from maternal blood samples. Decreasing concentrations over a period of 15 days (two samples at 2 weekly interval) is indicative of fetal death while the presence of significantly higher concentrations than controls at Day 50 appeared to be correlated with impending fetal death [45]. It has not been possible, however, to determine threshold concentrations that could be predictive of fetal demise in the clones, since the concentration of PAGs is consistently higher in SCNT pregnancies by Days 60-64 of gestation [17,24].

3.2 Prenatal monitoring - Identification of the high risk pregnancy

Late fetal loss occurs in 100% to 0% of SCNT pregnancies, depending on the breed, nuclear transfer technique and donor cell genotype and lineage, with an average figure of about 25-50% of pregnancies [46,64,70,72,91]. Fetal loss is associated with placental abnormalities leading to severe fetal hydrops, therefore necessitating rigorous monitoring of pregnancies in order to electively terminate pregnancies before maternal and fetal suffering become

an issue [16]. This is particularly important as ethical concerns regarding the welfare of fetal and neonatal clones as well as clone recipients have been the basis of the current debate leading to the postponement of the adoption of the European directive for Novel Foods [25], based on reports from the European Food Safety Agency and the European Group on Ethics in Science and New Technologies [30].

High risk pregnancies can be identified by external examination and clinical observations in conjunction with ultrasonographic imaging to detect abnormal edematous placentation and increased allantoic fluid, and clinical tests to detect urinary ketones and elevated maternal plasma PSP60 concentrations [16,48,64]. Regular trans-rectal palpation of the uterus can also be used to detect abnormally rapid fluid accumulation and edematous fetal membranes. When the uterus becomes atonal, the situation is critical [16]. Trans-abdominal ultrasonography is recommended for monitoring fetal viability and as an aid in detecting increased allantoic fluid volumes. Fetal heart rate has not proven to be useful for diagnosing fetal distress, as heart rates could not be measured in very severe cases where there was so much fetal fluid that it became impossible to locate the fetus [17,20,23]. Moreover, long periods of observation were not possible and continuous monitoring may be needed [13,55,68]. More research is needed as abnormalities in the fetal heart rate have been reported in IVP bovine fetuses as well [11]. In addition, although aortic diameter has been reported to be related to fetal size in the horse [1,75], it was not very useful for diagnosing very large SCNT bovine fetuses because even normal-sized SCNT fetuses may have enlarged hearts, thus making interpretation of the measurements difficult. The presence of placental edema or excessive allantoic fluid often indicates a poor prognosis for the fetus. The monitoring of the ultrasound appearance of placentomes is one of the most helpful tools for establishing a diagnosis of LOS. The edematous appearance, rather than the size of the placentomes (Figure 2), is indicative of the placental pathology in SCNT. Fetal ascites, which is often associated with abnormal placentation in SCNT, can also be monitored. In sheep, the fetal abdomen was punctured under trans-abdominal ultrasonographic guidance to retrieve the abdominal fluids from an SCNT fetus (Figure 3), but the abdomen filled up rapidly again and this procedure did not prevent fetal demise (P. Chavatte-Palmer and O. Picone, personal observations).

Monitoring of maternal body weight, feed intake, abdominal circumference, heart/respiratory rate and body temperature also provides valuable data to assess the health of the recipient animal. It has also been observed that some cows carrying SCNT fetuses have a fever of unexplained origin during the last few weeks of gestation. The presence of ketonuria is not necessarily a bad prognostic sign, but it does warn of potential for trouble. Significant reduction in feed intake or rapid gain in body weight or abdominal circumference indicate the development of hydrallantois and are prognostic of a poor outcome for the fetus.

Although at present there is no useful therapy for improving the prognosis for severe cases of hydrallantois, less severe cases may be nursed to term by paying careful attention to the feed intake and metabolic status of the dam. Ruminants in particular are very prone to metabolic disorders (ketosis and fatty liver) caused by the increased uterine volume and reduced rumen capacity that accompany hydrallantois. In any case, a differential diagnosis for enlarged abdomen is always multiple fetuses when multiple embryos have been transferred. Rapidly growing SCNT fetuses may easily cause metabolic problems in ruminants and regular monitoring of urinary ketones will allow early intervention. The gestational age at the onset of the pathology is an important prognostic indicator of outcome: in our laboratory, we consider that the fetus is likely to survive to term if the pathology manifests itself in the last two weeks prior to term. In severe cases, however, although the cow may be delivered with a live fetus close to term, the survival of the offspring is often compromised.

Poorly viable third trimester fetuses may have significant circulatory abnormalities (hypoxia, liver congestion, placental edema). This is likely to be secondary to inadequate placental development and thus the placental circulatory system requires more careful evaluation. As abnormal placental circulation would place undue circulatory and metabolic stresses on the fetus, prospective studies on SCNT pregnancies are currently being performed to evaluate placental blood flow with 3D power Doppler ultrasound after validating the method in the sheep (Figure 4) [67].

IV. UNDELYING MECHANISMS

Although sometimes, gross morphology appears normal in the placentomes of SCNT fetuses, many observations indicate that placental function

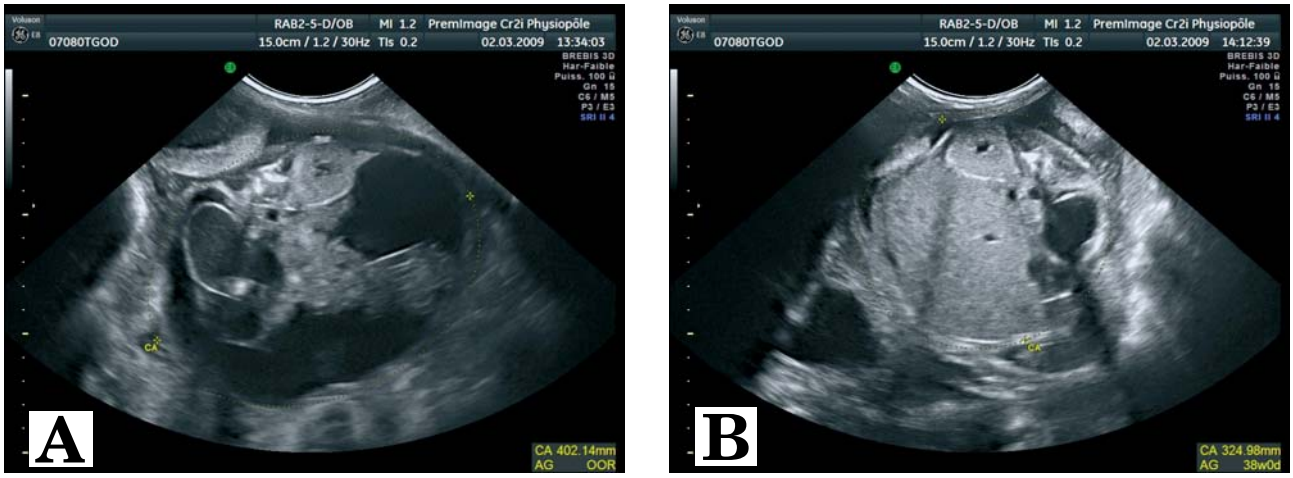

Figure 3. Coronal view of a cloned goat fetus with abdominal ascites (Voluson V8, GE Medical Systems, 3.5-5 MHz probe). A) before trans-uterine abdominal puncture, B) after trans-uterine abdominal puncture.

may be impaired or modified. The overall mean surface area of placentomes has been shown to be increased in abnormal SCNT pregnancies and differences in placentome types have been observed [9,12]. Microscopically, stereological analyses of histological sections of placentomes have shown that the proportion of fetal connective tissue was increased and that of maternal epithelium decreased in SCNT. It seems as if these structural modifications were occurring to increase the efficiency of maternal-fetal exchanges [23].

4.1 Placental vascularization

Enlarged and prominent placental and umbilical blood vessels have often been observed in SCNT fetuses. The study of the expression of genes involved in vasculogenesis has shown that the two major factors controlling neovascularization in the placenta were affected in bovine SCNT, in a sexually dimorphic way. VEGFR-2 was overexpressed in the placenta of SCNT males and bFGF expression was decreased in the placenta of SCNT females when compared to controls [14].

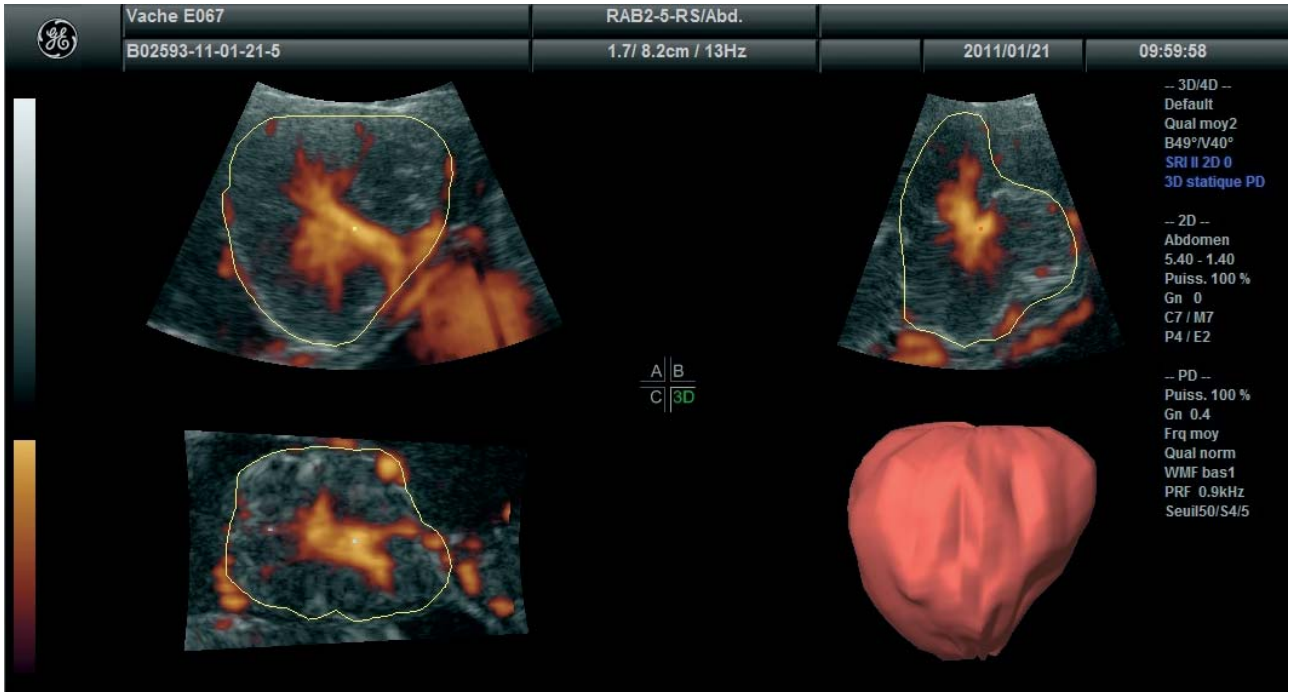

Figure 4. Transabdominal 3D power Doppler view (Voluson I, GE Medical Systems) of a placentome (ABC: power doppler view from 3 different planes, 3D: 3D reconstruction of the total volume of the placentome) in a cloned pregnancy. Evaluations are currently being undertaken to evaluate the interest of such a technique for the diagnosis of abnormal placental function in clone cattle.

4.2 Placental function and exchanges

The placenta is an organ that exists for the duration of the gestation solely to provide for the needs of the developing and growing fetus. Key functions would be the supply of adequate nutrients to the fetus, removal of fetal waste products, gaseous exchange, production of pregnancy-specific hormones and protection of the fetus against pathogens.

SCNT placental function is compromised as late gestation cloned fetuses and newborn calves have been found to be hypoxic [36], hypoglycemic and hyperfructosemic [8,9] and with a certain degree of anemia [8,19], the latter being possibly related to the placental edema observed in pathological pregnancies, as observed in humans [26,78]. Moreover, the analysis of the fetal/placental weight ratio also indicates that placental efficiency is reduced in late pregnancy (Figure 5) [23]. As a result of dysregulated placental development, a delicate balance exists between the capacity of the placenta to supply nutrients and the demands of a rapidly growing fetus.

All placental functions themselves require large amounts of energy. An important energy source for the growing fetus is glucose, which is transported from the maternal circulation through the placenta to the fetus. Biochemical analyses of fetal fluids in one study at Days 50, 100 and 150 of gestation showed that in general, the mean glucose values were not different between SCNT and normal pregnancies, except at Day 50 of gestation, when the mean amniotic fluid glucose concetrations were lower in SCNT [60]. Fetal membrane weights of these SCNT pregnancies were significantly higher at day 50 when compared with the controls and this may account for the greater utilization of the glucose, making less available to the fetus. However, once the placenta was formed, it appeared that glucose supply to the SCNT fetus was not significantly different compared with pregnancies generated by artificial insemination (AI) or with IVP embryos, as determined by mean amniotic fluid glucose levels. However, fetal fluid glucose levels in apparently failing SCNT pregnancies at Day 100 were unusually low, particularly in pregnancies with exceptionally large placentas or, in one case, a developmentally-retarded fetus with only 36 placentomes. Placental tissues themselves use 60-75% of the glucose leaving the uterine circulation and, with the excessive overgrowth of the SCNT placenta in the second half of gestation, the large placental mass could out-compete the fetus for available glucose. Glucose metabolized by the placenta and fetus contributes to a large part to the lactate present in fetal fluids and plasma. Highly elevated lactate levels, accompanied with reduced glucose and fructose concentrations, were seen in

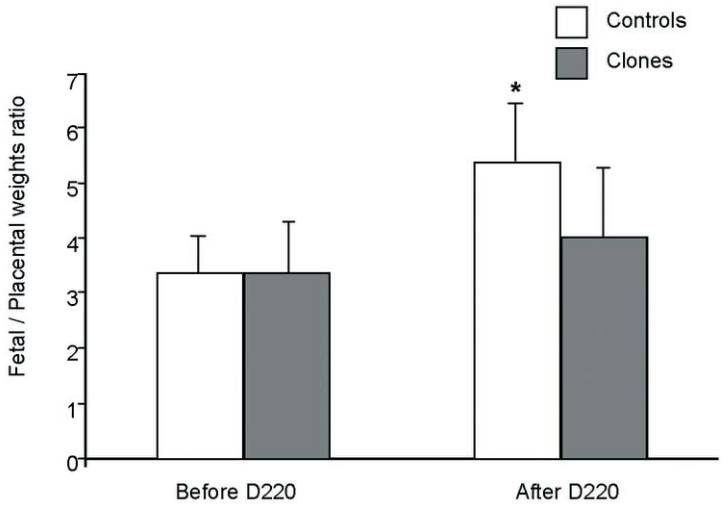

Figure 5. Mean ± SD feto/placental weight ration in control and clone pregnancies between 180 and 220 days (N=5 controls and N=9 clones) and after 220 days of gestation (N=10 controls and N=9 clones) (after [24]). *indicates a significant difference ($P < 0.05$).

allantoic fluid from SCNT fetuses with enlarged placentas and in some hydrops cases.

At term, the expression of the glucose transporter GLUT3 is increased in the SCNT placenta, and there is a negative correlation between maternal blood glucose prior to parturition and birth weight, in the absence of neonatal hypoglycemia, indicating that glucose uptake from the maternal circulation and transfer to the fetus is increased in late gestation in SCNT, probably to meet increased fetal requirements [51].

Another function of the ruminant placenta is to synthesize fructose from glucose. Generally, the ability of the SCNT placenta to synthesize fructose did not appear to be impaired. However, some allantoic fluid samples from SCNT pregnancies did have lower glucose and fructose and these pregnancies were associated with increased placental and fetal weights, which imply that placental fructose synthesis in these cases was being limited by glucose availability at this stage [60]. In contrast, at term, hyperfructosemia in SCNT neonatal calves at birth seems to indicate either increased production or decreased utilization near term [9].

The bovine placenta is very important source of steroid hormones during pregnancy [22,86]. Although it has the potential to produce both progesterone and estrogens, it does not have to capacity to produce enough progesterone in the absence of a corpus luteum to support the pregnancy until after 200 days of gestation. The placenta, however, is the main source of estrone and there are two stages in gestation when estrone production is up-regulated. The first rise in estrone levels takes place at the beginning of the second trimester. It is crucial for pregnancy progression. Retrospective analysis of maternal plasmas showed that the majority of SCNT pregnancies that fail around mid-gestation did not have this rise in estrone levels (Morrow and Lee, unpublished observations). In addition to possible effects on fetal development, estrogens also likely affect placental development and function, as estrogen receptors were found to be present in placental tissues [53].

The second rise in estrogen levels take place near the end of the gestation and one function for these estrogens is the preparation of the mammary gland for lactation. We have observed that those recipient cows with SCNT fetuses that showed no signs of preparation for parturition also show poor mammary development near term. This could be due to inadequate estrogen production or excessive metabolism of estrogens, and it is likely to be associated with the absence of a prepartum decline in maternal progesterone concentrations, as observed in a few cases [48]. The placenta regulates the amount of estrogens that both itself and the fetus are exposed to, by expressing estrogen sulfotransferase, which catalyzes the sulfoconjugation of estrogens for excretion. Excess sulfoconjugation was found to be a possible cause for the poor parturition signal frequently encountered in bovine SCNT pregnancies [52].

An abnormal increase in the concentration of PAGs has been reported in maternal circulation of SCNT recipients that subsequently developed placental anomalies [45]. PAGs, secreted in maternal blood, are synthesized by trophoblastic binucleate cells (BCN) that migrate and fuse with uterine epithelial cells, forming short lived trinucleate cells [94]. In cattle, other proteins such as placental lactogens (PL) are also produced by the BNC [95]. In a recent study, we reported that maternal plasma PAG concentrations were not different at Day 32, but significantly higher in SCNT than in AI and IVP control pregnancies at Day 62 and during the third trimester. Circulating bPL concentrations were undetectable at early stages and were not different in the third trimester between clone and control pregnancies. Placental tissular ratios of PAG or bPL to total proteins were not different between the two groups at all stages studied. Moreover, there was no difference in the percentage of PSP60-positive BNC in placental tissues between SCNT and control pregnancies. These results demonstrate that highly elevated maternal levels of PAG during abnormal SCNT pregnancies do not result from the placental hypertrophy, nor from an increased expression of the proteins by the placenta or a higher proportion of BNC, but could be due to changes in the composition of terminal glycosylation which result in a decrease of the clearance of PAG from the circulation [24].

In the bovine placenta, approximately 15-20% of the trophoblast is composed of giant trophoblastic cells that can have two or more nuclei (BNC, trinucleate cells..). It was shown that there is a lesser proportion of diploid to tetraploid cells in the central region of placentomes and in microplacentomes (<1 cm diameter). Fewer apoptotic cells were present in the central region of the placentomes and in interplacentomal regions and a greater proliferation capacity was found in all regions near term [76] in SCNT placentas, which may be related to the higher proportion of trophoblastic tissue

compared to maternal tissue in late gestation, abnormal, SCNT placentas [23].

4.3 Oxidative stress

In pigs, available evidence shows that aberrant expression of antioxidant enzyme proteins and associated oxidative stress in the extra-embryonic tissue derived from of Day 26 SCNT conceptuses is a major factor contributing to low birth rate [15]. Oxidative stress during early placental development is associated in other species with pregnancy-related disorders and pathologies in humans, such as embryonic resorption, spontaneous abortion, intra-uterine growth restriction, fetal death, preeclampsia, and preterm labor and delivery [2,54,87]. Adequate placental antioxidant status during early pregnancy could prevent those disorders induced by oxidative stress that lead to impairment of placental function and development, fetal growth and poor pregnancy outcomes. Indeed, the content of malondialdehyde (MDA), as an index of Reactive Oxygen Species (ROS) oxidative stress, is significantly lower in the chorionic tissue of bovine SCNT compared to AI controls at Day 62 of pregnancy [3]. Similarly, although the activities of the key anti-oxidant enzymes Super Oxide Dismutase 1 and 2 (SOD1 and 2) and Glutathione Peroxidase (GPX), were not different between the chorion of AI and SCNT conceptuses, at Days 32 and 62 of gestation, catalase (CAT) activity was significantly decreased at Day 32 and significantly increased at Day 62 in SCNT chorion, demonstrating that the activity of some of the anti-oxidant enzymes is up regulated in the chorion of post-implantation SCNT fetuses [3].

4.4 Gene expression

To understand how placental development in SCNT pregnancies is different from AI or IVP pregnancies, several gene expression profiling studies have been carried out. Different microarray/macroarrays with different sets of genes have been used in these studies, making it difficult to compare the findings between studies. In addition, the stochastic nature of the abnormalities, the different SCNT procedures used, and the stage of gestation at which the placental samples were collected and how the tissue was sampled makes it difficult to find sets of genes or pathways that are commonly affected. This is reflected in the microarray analysis of placenta collected from AI, IVP and SCNT pregnancies [31], where cluster analysis revealed that most of the variation in gene expression can be accounted for by the stage of gestation (pre-term versus term), the source of the placental samples (AI, IVP and SCNT) and the fetal pathology. However, the compound groupings in each cluster indicate that many factors contribute to the gene expression patterns. Interestingly, the majority of the AI term samples fell into a unique cluster that excluded IVP and SCNT placenta. IVP samples clustered more with the SCNT than with the AI samples, consistent with earlier observations that the placentas from IVP pregnancies, like those from SCNT, show greater gross morphometric variability than AI placentas [58]. This indicates that the environment of the pre-implantation embryo can have long-term effects on placental development and gene expression profiles. Many of the differentially expressed genes and overlapping functional networks among the IVP and SCNT gene lists support the hypothesis that early embryo culture conditions likely affect the same pathways in both SCNT and IVP. However, SCNT itself affects the expression of an additional > 200 genes that ultimately may contribute to the abnormal placental phenotype seen only in SCNT. The most prominent functional effects involve cell cycle, cell signaling pathways, molecular transport, DNA replication, recombination and repair; most of these molecular pathways and functions appear to be down regulated in SCNT.

Other studies have looked for differences in gene expression at earlier stages of gestation (peri-implantation and in the early stages of placentome formation), prior to the development of severe pathologies but before the early losses have occurred. However, these studies focused only on the extra-embryonic membranes or cotyledonary tissues and therefore cannot be compared directly with studies using tissues from entire placentomes. Several genes expressed in the trophoblast have been found to be differentially expressed between SCNT and control conceptuses, including placental lactogen (PL), the PAG, prolactin related protein-1 (PRP-1) [41] and Dickkopf-1(DKK-1)[57], to name a few. All these proteins are expressed in the BNCs of the trophoblast and thus, indicate that BNC function may be affected in SCNT from very early in gestation, thereby compromising placental development. At around Day 60 of gestation, the expression of two of the hemoglobin genes (HBA1 and 2) and the erythrocyte spectrin beta gene (SPTB)

was found to be down-regulated [71]. Reduced expression of these three genes could mean that erythropoiesis could be impaired in the SCNT placenta and thus, compromise fetal survival.

4.5 Epigenetics and the role of imprinted genes

Cloning by nuclear transfer returns a differentiated cell to a totipotent status, a process termed nuclear reprogramming, and was often associated with epigenetic alterations. Several studies have shown that insufficient epigenetic reprogramming of the differentiated nuclear donor cell towards a pluripotent (embryonic) status plays a major role in the pathogenesis of LOS [69]. Both global- and gene-specific DNA methylation has been studied in SCNT embryos and offspring of several species including mouse, pig, buffalo and cattle. In the early stages of development, a hypermethylation of the entire bovine embryo has been reported using immunostaining techniques, mainly revealing the methylation patterns of repetitive regions and heterochromatic regions of the genome [81].

Many of the pathologies reported with bovine clone pregnancies resemble abnormalities reported with either mutations or deletions of imprinted genes or dysregulation of imprinted gene expression. Embryo manipulations, such as assisted reproductive technology (ART), induce phenotypic changes in the embryo and the placenta by influencing imprinting genes, most of which being expressed in the placenta, in mouse [63], sheep [97] and human [4,34]. Studies in mouse mutants have shown that many of the genes involved in differentiation and growth of the placenta are imprinted [42,79]. Examples of imprinted genes which control placental growth include Igf2r [88], Igf2, Mash2 [39], Esx1 [61], Cdkn1c/p57^{Kip2} [85] and Phlda2/Ipl/Tssc3 [35].

At the blastocyst stage, alteration of the epigenetic regulation of some imprinted genes is often associated with the embryo death. A loss of DMR methylation (differentially methylated regions implicated in the imprinting regulation) was found for IGF2R [62] and SNRPN [84] genes. In contrast, an overall lesser methylation in the DMR of the IGF2/H19 alleles was found in cattle when compared to the same tissues in control animals [37]. In the mouse, overexpression of Igf2 results in fetal and placental overgrowth and overgrowth of certain fetal organs [29]. The same organs are often the ones that are disproportionately large in SCNT fetuses. Although overexpression of IGF2 (with reference to house-keeping genes) was not observed in placental tissues from SCNT pregnancies, the increased placental mass means that overall, there must be more IGF2 produced and available to promote growth, assuming there is no difference in translational efficiency.

The bioavailability of IGF2 is regulated through binding to the type 2 receptor (IGF2R) or to the IGF binding proteins (IGFBPs). The expression of IGF2R was shown to be altered in ovine large offspring syndrome [97]; however, this was not the case in bovine clones. Aberrant expression of the IGFBPs in bovine SCNT was reported [74], and even though the genes for these proteins are not imprinted, they regulate IGF2 bioavailability at a tissue-specific manner. The expression IGFBP2 was lower in the placental tissues from SCNT pregnancies [59]; thus, even if IGF2 production was not increased in clones, there was greater availability, as there was less IGFBPs to bind to it.

The maternally expressed Phlda2 gene is included in a cluster of imprinted genes located on mouse chromosome 7, synthenic to a cluster on human chromosome 11p15.5 [73]. Phlda2 invalidated mice, with a lack of the maternal expression of Phlda2, are fertile but present enlarged placentae during pregnancy [35]. Conversely, the loss of imprint of Phlda2 leading to a bi allelic expression, causes fetal and placental growth retardation [80]. Similarly, an increased expression of PHLDA2 in human placenta is associated with low birth weights through putative reduction in size and function of the placenta [5]. In bovine, a reduced expression of Phlda2 is associated with pathological overgrowth of the placentomes from SCNT pregnancies and the level of expression of PHLDA2 is correlated negatively with total placentome weight in cloned placenta [40]. Altogether these data strongly support the idea that PHLDA2 acts as regulator of placental growth and may be involved in overgrowth observed after SCNT.

Imprinted genes may regulate fetal growth by affecting the overall development and growth of the placenta or its functions, such as the supply of nutrients to the fetus via specific transporters and ion channels. Some of these transporter genes, such as the SLC22A genes are linked to IGF2R. It is not known if the expression of the SLC22A genes is affected in the SCNT placentas. Although a larger placenta may have a greater surface area over which to transfer nutrients, aberrations in vascular development may alter permeability and the increased mass, in addition, requires a greater share of the nutrients to maintain function. Placental mass is not always positively correlated with fetal weight in bovine

SCNT pregnancies, so a fine balance between supply and demand is required to maintain an appropriate rate of fetal growth.

In female mammals, one of the two X-chromosomes is randomly inactivated in somatic cells in the body. Some SCNT embryos, aborted fetuses or dead newborn calves or viable offspring, however, showed aberrations in X-chromosome inactivation [96]. This has been attributed to faulty reprogramming of the donor nucleus. In the placenta, which normally show preferential paternal X-inactivation due to imprinting, random X-inactivation has been reported in SCNT placenta. It has been postulated that this aberrant X-inactivation may be deleterious to the development of SCNT fetuses or contribute to abnormal placental development. However, we did not see any gross differences in the severity of gestational pathologies, either in the placenta or fetus, whether male or female donor cells are used (Lee, *et al.*, unpublished observations). There should not be any issues with X-inactivation when male donor cell lines are used, yet the outcome is no better compared with using female cell lines. Thus, aberrant X-inactivation is unlikely to be a major contributor to poor cloning outcomes.

Finally, it must be noted that a high variability of global methylation has been reported in healthy adult clone cows [27]. Altogether, these studies highlight the importance to analyze the methylation status of specific loci in cloned embryos and offspring to understand the epigenetic disruption associated with the SCNT procedures.

V. CONCLUSIONS

In conclusion, SCNT affects many aspects of placental development in the bovine species, from the morphological structure to physiological function, gene expression and epigenetic marks. It is now well recognized that events occurring in the embryonic and fetal period may induce long term health effects in the offspring as developed through the study of the Developmental Origins of Health and Disease (DOHaD) [7,10,38]. Studying long term effects of cloning therefore must encompass the study of the effects of nuclear transfer *per se* as well as the secondary long term effects due to a perturbed placental function.

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