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Placental Insufficiencies in Cloned Animals – A Workshop Report

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Abstract

This workshop focused on describing clinical problems identified in the placentae of cloned animals and some of the potential biological mechanisms by which these anomalies arise. It was shown that placental anomalies related to somatic cell nuclear transfer (SCNT) in cattle often can be detected by ultrasonography early in gestation, enabling preventive clinical intervention. On the mechanistic front, the vascular defects in the placenta appear to be associated with anomalies in the expression of VEGF system, which could lead to the aberrant placentomes and generalized oedema seen in some gestations. Moreover, an upstream transcription factor (Mash2) controlling the differentiation of trophoblast into binucleate cells may be involved in the poor implantation rates of SCNT embryos. Finally, epigenetic patterns in placenta can be disrupted by fairly simple *in vitro* manipulations, which could explain the extreme anomalies observed in the placenta of SCNT pregnancies.

Keywords: Cloned animals; Placenta; Epigenetics; Genomic imprinting; Mash2; VEGF-A; Somatic cell nuclear transfer

1. Introduction

Our ability to produce clones by somatic cell nuclear transfer (SCNT) has improved significantly in the last decade. However, the majority of cloned animals are lost either during gestation, because of failure to produce a functional placenta, or due to neonatal complications originating from an abnormal placental environment. The major macroscopic and microscopic alterations in placentation after SCNT have been characterized and some of the molecular mechanisms associated with the differentiation of placenta are beginning to emerge. Since effective methods of intervention to avoid or circumvent these complications remain elusive, our focus should be directed towards a better understanding of the roots of placental development in animal clones. Therefore this workshop focused on describing clinical problems identified in the placentae of cloned animals and some of the potential biological mechanisms by which these anomalies arise.

Ruminants have a cotyledonary placenta. Instead of a single large area of contact between maternal and fetal vascular systems, there are numerous placentomes, each comprised of a cotyledon (fetal side) and a caruncule (maternal side). A prominent feature of ruminant placentation is the presence of large numbers of binucleate cells, which arise from trophoblast cells that fail to undergo cytokinesis following nuclear division. They invade and fuse with caruncular epithelial cells to form small syncytia and secrete a number of pregnancy related proteins.

2. Clinical aspects of placental anomalies in cloned cattle

Rejean Lefebvre and Reza Kohan-Ghadr noted that, in spite of the potential advantages of assisted reproductive technologies in cattle, pregnancies derived from cloning technologies

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have been associated with aberrant placental phenotypes, health problems in newborns and surrogate mothers and high rates of pregnancy loss. SCNT is associated with poor reproductive efficiency (<5%) and is characterized by low pregnancy rates, high pregnancy loss and a plethora of fetal and placental abnormalities. The majority of established SCNT pregnancies in cattle are lost between days 30 and 90 of gestation, in association with poorly developed placentomes [1]. In the first trimester, placentomes appear to have abnormal shapes and are present in reduced numbers. If pregnancy is maintained, placentomes are frequently hypertrophied [2]. Anomalies appear more extreme in clones produced from somatic cells relative to those produced from embryonic cells [3]. Placentome length (Fig. 1) and umbilical diameter increased significantly more in clone-derived compared to normal pregnancies. In addition oedema of the fetal membranes, a large accumulation of allantoic fluid (hydroallantois), reduced epithelium at the endometrial–trophoblast interface and underdeveloped vasculature are observed [2,4]. The presence of inflammatory cells is accompanied by haemorrhage or by thrombosis of large chorioallantoic vessels. Ultrasonographic monitoring enables further characterization of the changes in the placenta and can be used to assess fetal well-being in clone-derived high-risk pregnancies in cattle. New studies of uterine artery blood flow will further increase our understanding of the presence of oedema in the fetal membranes and the accumulation of a large amount of fluid in the allantoic sac. A detailed clinical description of placental anomalies associated with cloned pregnancies may help to generate specific hypotheses as to their cause and increase the efficiency of SCNT.

3. Analysis of the VEGF-A system in the placenta of cloned cattle

Christiane Pfarrer and her co-workers, Danila Campos and Paula Papa, reported that the amount of fetal mesenchyme is significantly higher in cloned placentae [4]. Since vascular endothelial growth factor-A (VEGF-A) has been shown to be crucial for vasculogenesis and angiogenesis during embryonic and fetal development, it likely plays a role for vascular growth and permeability in the bovine placenta. Analysis of the VEGF-A system in near term placentomes from SCNT pregnancies and comparison with placentomes derived from artificial insemination (AI) pregnancies included detection of mRNA and protein of VEGF-A and its receptors VEGFR-1 and VEGFR-2. Preliminary results indicated that none of these components of the VEGF system differ significantly between placentae from cloned and AI groups. In contrast, a comparison between individual clones revealed a strikingly high variation in the levels of VEGF-A, VEGFR-1 and VEGFR-2 mRNA and protein in placentomes of SCNT fetuses. This variation correlated strongly with the intensity of immunostaining, suggesting a deregulation of the VEGF-A system in the placenta of bovine somatic cell clones which may contribute to the placental alterations and/or malformations reported. The cellular localization of VEGF-A and its receptors in the placentae of cloned cattle did not differ from that earlier observed in placentae developing after AI [5]. These findings corroborate the hypothesized role of the VEGF system for placental vasculogenesis and angiogenesis, which seems to be disturbed in placentomes of cloned fetuses.

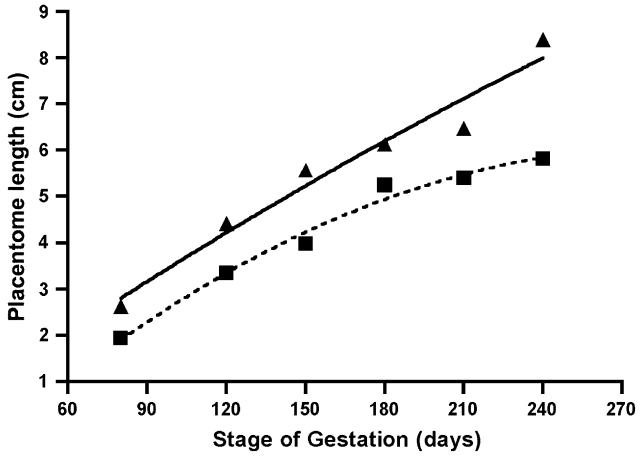

Fig. 1. Placentome growth in cloned pregnancies. Placentome size in cloned (triangle) and control (square) gestations at different periods of gestation. Lines represent nonlinear regression plots for cloned (continuous) and control (dotted) groups.

4. Effects of SCNT on trophoblast development in bovine embryos

Daniel Arnold and Lawrence Smith reported that, since fetuses produced by SCNT commonly possess placental abnormalities, it is plausible to infer that upstream differentiation pathways are affected in these pregnancies. For this reason, a series of experiments was performed to analyze trophoblast development (both cellular and molecular) during the peri-implantation period (days 17 and 40 of gestation) in *in vivo*, *in vitro* and SCNT produced bovine embryos [6]. The bovine trophectoderm is comprised of two cell types; mononucleate cells and binucleate trophoblast giant cells (TGC). Mononucleate cells make up the majority of the trophectoderm and are responsible for producing interferon-tau (IFN-t), the pregnancy recognition signal in cattle, between days 8 and 24. TGC make up 20% of the mature bovine placental trophectoderm and produce pregnancy associated proteins such as pregnancy associated glycoproteins (PAG). Utilizing these genes as markers at day 17 of gestation, IFN-t mRNA was not different among any of the groups. PAG-9 mRNA was highest in AI, reduced in *in vitro* fertilization (IVF) and not detectable in day 17 SCNT embryos. By day 40 of gestation, the placenta is in immediate contact with the uterus and cotyledons are starting to form. By morphology and immunostaining with an antibody specific for PAGs, SCNT cotyledonary tissue had less TGC than AI and IVF tissues. Two genes (Mash2 and Hand1) appear to play a critical role in trophoblast development of several species including the cow. For Mash2, a gene involved in mononucleate cell proliferation, mRNA is specifically expressed in bovine cotyledonary tissue. In

addition, expression of Mash2 mRNA is higher at day 17, when the rate of trophoblast proliferation is greatest. In SCNT embryos at day 17, Mash2 mRNA expression is two-fold higher than in AI embryos. Hand1 is involved in TGC formation and Hand1 mRNA expression was reduced in SCNT embryos compared to IVF and AI at day 17. At day 40, Mash2 and Hand1 mRNA were greater in SCNT than AI cotyledonary tissue. The overexpression of Mash2 was not due to altered imprinting, since in all groups Mash2 showed biallelic expression prior to implantation and monoallelic maternal expression at day 40. These results suggest that Hand1 may be overexpressed to compensate for the higher Mash2 expression, and may explain why later stage cotyledons are larger and have more TGC. To overcome the difficulty of obtaining tissues to study, Dan Arnold evaluated *in vitro* trophoblast cell cultures. Trophoblast cell lines from SCNT embryos replicated twice as fast as trophoblast cell lines from IVF embryos. In addition, Mash2 expression was higher in SCNT cell lines compared to IVF cell lines. Mash2 expression was biallelic for both groups suggesting these cell lines may be similar to day 17 trophoblast cells. These studies provide more insight into bovine trophoblast development and the alterations associated with SCNT. More Mash2 mRNA and fewer TGC suggest that SCNT trophectoderm does not develop normally and these effects may be one of the causes of pregnancy loss in SCNT bovine embryos.

5. The role of epigenetics in normal and abnormal placental development

Amanda Fortier described the phenomenon of genomic imprinting, which results in the parent-of-origin specific monoallelic expression of genes, and its potential implication in placental abnormalities. The correct epigenetic patterns are present in mature germ cells, so that following fertilization, the appropriate configuration of DNA methylation is contributed by each parent. These patterns must be maintained throughout development; however, the patterns are erased in the developing germ line and re-established according to the sex of the developing embryo. Both establishment and maintenance of epigenetic marks may be susceptible to disruption. Imprinted genes play an important role in embryo growth and development as well as in placental function, but there are several differences between the embryo and the placenta that include lower overall levels of methylation. While DNA methylation is very important for maintaining monoallelic expression of imprinted genes in the embryo, it appears that chromatin modifications may be of greater importance in the placenta [7]. In the mouse, SCNT involves several techniques including superovulation, embryo culture and embryo transfer protocols. The failure of placental development following SCNT is believed to be the major reason that cloning has a low success rate. It is known that many imprinted genes are important for the development of the placenta. It has been shown that even relatively minor manipulations, such as embryo culture, can affect expression of imprinted genes in the placenta [8]. Further, it was shown that superovulation also affects expression of imprinted genes specifically in the placenta. These findings contribute to the evidence that mechanisms for maintaining imprinting are less robust in trophectoderm-derived tissues. It was suggested that extraembryonic tissue may be more tolerant of epigenetic disruption, perhaps because it is short-lived. Alternatively, the placenta may be more susceptible to epigenetic dysregulation, which may be an evolutionary mechanism to prevent the development of embryos that have been exposed to unfavourable conditions during early development. Given that embryo culture and superovulation alone can affect the expression of imprinted genes in the placenta, the full protocol involved in SCNT is likely to cause much greater perturbation.

6. Conclusion

This workshop clarified the placental phenotypes observed in cloned animals and identified potential causes of the placental insufficiencies. Nonetheless, it is clear that much more needs to be learnt about the molecular mechanisms to elucidate the origin and the potential preventive treatment for SCNT-derived placental defects and to enable a better gestational outcome for cloned animals.

7. Conflict of interest

The authors do not have any potential or actual personal, political, or financial interest in the material, information, or techniques described in this paper.

References

[1] Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, et al. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 2000;63:1787–94.

[2] Constant F, Guillomot M, Heyman Y, Vignon X, Laigre P, Servely J, et al. Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. Biol Reprod 2006;75:122–30.

[3] Heyman Y, Chavette-Palmer P, LeBourhis D, Camous S, Vignon X, Renard JP. Frequency and occurrence of late-gestation losses from cattle cloned embryos. Biol Reprod 2002;66:6–13.

[4] Miglino MA, Pereira FT, Visintin JA, Garcia JM, Meirelles FV, Rumpf R, et al. Placentation in cloned cattle: structure and microvascular architecture. Theriogenology 2007;68:604–17.

[5] Pfarrer C, Raziwa S, Winther H, Callesen H, Leiser R, Schams D, et al. Localization of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2 in bovine placentomes from implantation until term. Placenta 2006;27:889–98.

[6] Arnold DR, Bordignon V, Lefebvre R, Murphy BD, Smith LC. Somatic cell nuclear transfer alters peri-implantation trophoblast differentiation in bovine embryos. Reproduction 2006;132:279–90.

[7] Lewis A, Mitsuya K, Umlauf D, Smith P, Dean W, Walter J, et al. Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet 2004;36:1291–5.

[8] Mann M, Lee S, Doherty A, Verona R, Nolen L, Schultz R, et al. Selective loss of imprinting in the placenta following preimplantation development in culture. Development 2004;131:3727–35.