



Workshop: III Symposium of the South American Research Consortium on Cloning and Transgenesis in Ruminants

## **Molecular strategies for gene modification in livestock**

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**Keywords:** genetic engineering, genome editing, transgenesis.

The use of reproductive biotechnologies in livestock over the past 50 years has had a productive impact of enormous magnitude, with great consequences on food production in terms of quality and quantity. One of the biotechnologies developed in the last two decades, transgenesis, is positioned as a set of technologies applied to animal assisted reproduction. This technique emerges as a tool that goes beyond animal production, as it not only includes these aspects, but also incorporates animals as source of food with special qualities (nutraceuticals) and/or of drugs for animal and human health (pharmaceuticals). Biopharmaceutical production is useful for a variety of purposes, including the expression of enzymes important in human nutrition. Until 2012 transgenic domestic animals were produced mainly by inserting a vector containing the gene or genes of interest in cultured donor cells for somatic cell nuclear transfer (cloning). Currently, new genetic tools are being developed and allow the manipulation of the genome, making more efficient mutation events directed by a cut in the double-stranded DNA. As a result, the cell recruit endogenous repair machinery, and this can be repaired by homologous recombination or non-homologous end joining, thereby generating a reliable arrangement, a deletion or insertion of nucleotides. The last two options produce a change in the open reading frame generating premature stop codons in two thirds of cases. Normally this type of transcript is degraded by a mechanism known as Nonsense Mediated Decay (NMD). One such tool, CRISPRs-Cas9 comes from bacteria and once inside the plant cell are capable of activating genes, thus creating better conditions for their survival and replication. It has been shown that artificially designed CRISPRs are able to recognize and bind to specific sequences and are capable of activating the transcription of specific genes, thus opening the door to a wide variety of applications in genome engineering.



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## **Transgenic animals for the production of therapeutic proteins in the milk**

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**Keywords:** bioreactors, genetic engineering, livestock.

Therapeutic proteins have a long history, initially being extracted from animals or even from human sources. With the approval of recombinant human insulin in 1982 by FDA, the first approved therapeutic recombinant protein, a huge market for recombinant therapeutic proteins was envisioned. Since then, more than 200 recombinant drugs have been approved in the USA and European Union, representing an increasingly important group of pharmaceuticals. The term biopharmaceuticals started to be used in the 1980s to define products generated by recombinant DNA technology or genetic engineering, which has dominated the biotech industry in the last 20 years. More specifically, the recombinant production of therapeutic proteins for the treatment of complex human diseases is a major force linked to transgenic animal production. This class of drugs is currently the largest source of innovation in the pharmaceutical industry. Over the past decade, the market for biopharmaceuticals maintained a steady growth of around 13.3% per year accompanied by a 4.2% decrease in the same period for other types of drugs. In addition, biopharmaceuticals account for 55% of all new approved drugs and 64% of drugs in development and/or approval. The marketing growth became the driving element of all efforts related to the development of new therapeutic proteins of biological origin, in which, the transgenesis can constitute a key component. Among the systems used in the search for production of biopharmaceuticals, the development of animal platforms based on the use of transgenic animals offers particularly attractive possibilities. The main advantages lie in low production costs combined with high productivity and the quality of the synthesized proteins. Currently, the recombinant protein expression in milk is the most robust system for the production of biopharmaceuticals from transgenic animals. Within this scope, transgenic goats have been widely used due to several advantages. The expression system in the mammary gland is the only one within the animal platform to generate recombinant proteins as legalized trade for therapeutic use, as Atryn<sup>®</sup> (rREVO Biologics Inc), a recombinant antithrombin produced in the milk of goats, was the first biopharmaceutical produced from a transgenic animal approved for human treatment in 2006 by the EMA (European Medicines Agency) and in 2009 in the US-FDA (Food and Drug Administration). More recently, rabbits have joined the goats in the approval of another biopharmaceutical from milk, Ruconest<sup>®</sup> (Pharming), a recombinant human C1 esterase inhibitor protein (C1INH). Following a similar path and trend, Ruconest was first approved by EMA in 2012, followed by the FDA approval in 2014. This revolutionized the landscape of scientific and marketing biopharmaceutical possibilities and added much to the reliability of recombinant protein production system through animal platform using the expression system in milk. We have been engaged in recent years in the establishment and development of *in vitro* and *in vivo* studies using transgenic animal models (goats, cattle) to produce animal milk containing therapeutic proteins to be used as biosimilar drugs and also to produce recombinant vaccines for use in humans and animals. The mammary gland transgene expression systems for the production of functional proteins in the milk of animals have been proven as a viable technological alternative to aid in the resolution of problems of the modern world.



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## Conceptus metabolism and systemic physiology in cloned cattle

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**Keywords:** Abnormal Offspring Syndrome, *in vitro* fertilization, somatic cell nuclear transfer.

Improvement of fertility in agriculturally-important species will have a direct impact on key segments of the livestock industry and agribusiness around the world, as well as on the environment and animal well being. One of the most critical aspects of infertility in production animals is the failure of a female to deliver viable offspring. That scenario is a typical feature of intensive production systems, as in dairy cattle, for which such failures may exceed 60% from ovulation to term. Lower pregnancy rates with higher gestational losses result in lower prolificacy, and represent a huge economic loss. This is an example of where basic and applied science should converge to the benefit of knowledge and the livestock industry. *In vitro* embryo production (IVP) in livestock species by *in vitro* fertilization (IVF) or cloning by nuclear transfer (NT) procedures have been of extraordinary importance for research and development, providing new knowledge, insights, and strategies for the resolution of fertility problems. In such scenario, Brazil has been leading the commercial IVF activity in the world, by the transfer of more than 300 thousand IVF embryos a year. However, the IVF and NT technologies are often associated with increased rates of pregnancy losses, developmental abnormalities, and birth of large and abnormal offspring with lower postnatal survival. As the Abnormal Offspring Syndrome (AOS) limits the practical use of some *in vitro* technologies, and as the physiological basis for the abnormalities is still widely unknown, IVF and cloning procedures provide excellent tools for the study of many biological processes that need to be elucidated in order for abnormalities following *in vitro* embryo manipulations can be fully understood. Recent changes in IVP systems for IVF procedures, especially for *in vitro* embryo culture conditions, have been minimizing problems, with conceptus traits in IVF-derived concepti and newborns getting to be similar to controls, with slight changes in placentome development that does not seem to affect conceptus growth and survival. However, cloning by NT continues to unpredictably result in abnormal phenotypes and higher morbidity and mortality after birth. In a systematic study on the AOS in cattle, we have shown significant differences in conceptus development between *in vivo*-derived and IVP pregnancies, including fewer but larger placentomes and increased placental surface area during late pregnancy, increased glucose and fructose accumulation in fetal fluids, with a seemingly effect on life *ex utero*. Pregnancy outcome may be similar to controls, but pregnancy losses in the first trimester can be greater than 70%. A biphasic growth pattern is commonly seen, with early conceptus being initially smaller than controls, but significantly larger and heavier in late pregnancy and after birth. Usually, a higher frequency of enlarged placentomes (more than 60%) may be observed after cloning, in an apparent greater role of less frequent and abnormal, nonetheless heavier, placentome types in placental function. We have associated the occurrence of enlarged IVP concepti with changes in metabolic and molecular profiles in the fetus and/or placenta in late pregnancy and after birth. Significant difference exists in activity in metabolic pathways and placental function in cloned concepti, suggesting an active glucose synthesis, an increase in fructose synthesis by the placenta, and in fructose catabolism by the fetus, which may be a reflection of an association between changes in metabolic fetal programming and excessive prenatal growth after cloning. In addition, *in vitro*-derived newborn calves may be heavier and larger at birth, having lower respiratory rate and thermoregulatory response than *in vivo*-derived controls, with higher concentrations of fructose in the immediate period after birth. In general, physiological and metabolic findings at birth may be widely similar and normal when compared with controls. However, during the first 24 h of life, IVF-derived calves showed physiological, metabolic, biochemical, hemogasometric, and hematologic features indicative of a lower adaptation to life *ex utero*, particularly in the first 4 to 6 h of life. The main differences indicate that larger animals, mainly IVP-derived calves, have more difficulties to maintain plasma oxygen levels, with evidence of a metabolic shift and elevation of metabolic substrates (mostly lactate), and a trend for acidosis followed by a compensatory normalization of the acid-base balance, predominantly in the first 6 h of life. Also, the fructose metabolic pathway was shown to be active in *in vivo*- and *in vitro*-produced newborn calves in the first hours of life. Consequently, the presence of pregnancy-derived higher fructose levels in the plasma of newborn calves may be metabolically and clinically beneficial for the postnatal adaptation of the life *ex utero* under uneventful conditions, but deviations from physiological normality, such as respiratory distress, may cause profound metabolic consequences that might compromise postnatal survival, leading to distress and death, an event more commonly seen in *in vitro*-derived newborn calves. In summary, morphological changes in cloned concepti likely influence placental function and metabolism, disrupting the placental constraining mechanism on fetal growth, leading to accelerated conceptus growth detectable in late pregnancy. Problems associated with cloning and IVF have already 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 the physiology of pregnancy, placental function, epigenetic reprogramming, transgenesis, neonatology, etc. Such research will continue to increase our understanding of developmental processes of physiological (e.g., placentation) or pathological (e.g., embryonic mortality) interest. Thus, new knowledge may become applicable in the future, with direct economical implications for livestock production, by providing new insights for ways to minimize losses and increase fertility in agriculturally-important species.



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## **Production of recombinant vaccines in the milk for human and animal diseases**

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**Keywords:** genetic engineering, transgenesis, vaccines.

Zoonotic diseases can be passed between animals and humans and are caused by a variety of organisms as viruses, bacteria, parasites and fungi. The spread of zoonotic diseases occurs at the human animal interface, which in most cases is the close contact between livestock and farm workers. In developing countries, the lack of proper sanitation and processing of food products increases the likelihood of disease transmission between livestock and humans. It is estimated that more than 6 out of 10 infectious diseases in humans are spread from animals. Moreover, increasing emergence of drug resistant strains of bacteria, stemming from improper use of antibiotics in human and veterinary medicine, as well as livestock production, escalate the risk to both humans and animals. The most effective means of eradicating zoonotic diseases would be to develop vaccination strategies that could be implemented simultaneously in both the human and animal populations, which is the essence of the One Health philosophy. Multivalent subunit vaccines have been utilized in a disease specific manner. However, there has never been a published attempt to produce a single vaccination platform containing multiple antigens to multiple pathogens that can be used in both humans and livestock. In such scenario, the transgenic animal platform of expressing recombinant proteins in the milk offers particularly attractive possibilities. The main advantages lie in the triad that combines the low cost of production with high productivity and quality of the produced proteins. Probably this triple alliance brings together the best attributes related to the success of any biopharmaceutical production platform. Recently, we have launched the first steps in the direction for setting up a production platform for recombinant vaccines in the milk of animals that allows us to quickly produce and test different antigens and allocate the best immune response to large-scale production using mammary gland as a bioreactor, offering protection from multiple pathogens in a single vaccine. By applying a transient approach, subunit vaccines can be screened and developed to target multiple pathogens and can be effective for both humans and animals. The mammary gland can efficiently produce large quantities of affinity tagged antigens capable of efficient low cost purification and production into a single effective vaccine for humans and animals. Furthermore, and perhaps most importantly, once the animals are genetically modified produced, they can be propagated, milked and the vaccines processed in the areas of need, which can further reduce cost and provide economic stability in developing regions, offering protection from multiple pathogens in a single vaccine. We believe that designing gene expression constructs capable of producing dozens of affinity tagged peptides offers the best approach for the development of a single vaccine that can block transmission of many devastating zoonotic pathogens anywhere in the world.



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### **Human and porcine induced pluripotent stem cells as disease models**

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**Keywords:** animal models, iPSC, stem cells.

Neurodegenerative disorders with Alzheimer's disease (AD) being the most prominent are worldwide increasing in frequency. We aim at establishing *in vitro* cell models as well as whole animal models for AD. We have refined a reprogramming strategy that allows for human fibroblasts from patients with AD to be reprogrammed into integration-free induced pluripotent stem cells (iPSC). During iPSC reprogramming the fibroblasts undergo a very well orchestrated mesenchymal-to-epithelial transition. The patient-specific iPSCs are subsequently differentiated into neurons, which express certain phenotypic characteristics of AD in the petri dish. The diseased neurons can be utilized for drug development. Furthermore, molecular tools as e.g. CRISPRs now allow for correction of mutations that cause the disease in familial cases. Hence, gene edited isogenic control cell lines can be established and strictly compared with the disease lines on the same genetic background. In the future, iPSC-based therapy for e.g. Parkinson's disease will be realized. A large animal model for studying the potentials and safety is needed. In order to pave the way for this, we have focused efforts on establishing porcine iPSCs as a model. We have tried different avenues and found that pluripotency transcription factors controlled by doxycycline-dependent promoters may drive temporary iPSC characteristics, which, unfortunately, fade when doxycycline is removed. However, using the plasmid-based reprogramming technology, applied to human reprogramming as above, we have been able to establish stable porcine cell lines with iPSC characteristics. Moreover, we found that a subfraction of porcine fibroblasts expressing the surface marker SSEA1 are particularly prone for iPSC reprogramming. In future projects we will also investigate canine cognitive dysfunction, a spontaneous condition in dogs with great similarities with AD, in order to develop the dog as a potential model for AD.

**Acknowledgments:** People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°PIAPP-GA-2012-324451 (STEMMAD), Innovation Fund Denmark (BrainStem and NeuroStem), UCPH Excellence Programme for Interdisciplinary Research (KU2016), Danish Council for Independent Research, Technology and Production Sciences.



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## **Perspectives and cloning outcome at In Vitro-Brazil Clonagem Animal S.A.**

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**Keywords:** equine, ICSI, somatic cell nuclear transfer.

Somatic cell nuclear transfer has been adapted from frogs for use in sheep as well as for use in many other mammalian species including bovine and equine. The birth of Brazil's first 3 cloned foals resulted from work performed while developing equine oocyte maturation, cell synchronization techniques as well as embryo culture and embryo transfer medium optimized for use in equine based off our current bovine cloning method which uses donor cells synchronized in G2 of mitosis with oocytes in Telophase II of meiosis. This cloning approach differs significantly from that used in the creation of Dolly. Our approach adapting bovine cloning methods for use in the equine was initially focused only on embryo culture media, maturation components and time of activation. This proved insufficient, as embryo development was poor and not suited for a commercial enterprise. We moved forward the following year by further optimizing our equine cloning protocols from oocyte maturation medium to equine embryo transfer medium and all procedures between. In Vitro Clonagem provides four products: complete services for bovine and equine cloning, equine ICSI as well as cell line establishment and cryopreservation. Each of our cloning services guarantees timely delivery of the cloned bovine or equine based on a contractual agreement. Cell line establishment guarantees the animal genetics can be safely preserved well after the original animal dies. At the present time, equine ICSI is the one service where we can send a pregnant recipient back to the owner's farm after 90 days. Thus far we are observing more interest in equine cloning and especially in equine ICSI. As IVF in equine is not yet a viable option due to low fertilization rates, equine ICSI represents an alternative for continued reproduction in older mares. As for now, we can only provide services to replicate genetics brought to us from clients. In the future, we hope to offer services that help advance genetic improvement by offering gene editing and IVF trophectoderm biopsy services with our parent company, In Vitro Brasil. Visit us at <http://invitrobrasil.com.br/en/cloning.php> or email me directly at [marc@invitrobrasil.com.br](mailto:marc@invitrobrasil.com.br) for more information.



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### **Phenotypic characteristics of F1 generation of transgenic goats producing hG-CSF in milk**

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**Keywords:** goats, recombinant protein, transgenesis.

The discovery of DNA has opened many areas of research including biotechnology and genetic engineering. The possibilities of isolation and introduction of genes into cells has become a routine procedure in laboratories around the world. Recombinant proteins of high economic value have been expressed in genetically-modified mammalian cells, which are better prepared for the synthesis of complex proteins. However, the need of large capital investment, high operating costs and relatively low production levels result in the inability to produce more than a few kilograms of protein per year (Houdebine. *J Soc Biol*, 203:323-328, 2009). Given these limitations, the transgenic animal platform in which the recombinant protein is expressed usually in their mammary gland and thus purified from their milk, appeared as a promising method due to some features, such as low operating costs and virtually unlimited capacity to scale-up by simply breeding transgenic animals (Kues and Niemann. *Prev Vet Med*, 102:146-156, 2011). Human granulocyte-colony stimulating factor (hG-CSF) is a cytokine of high economic value currently produced in bacterial and Chinese hamster ovary (CHO) cells for clinical use. This cytokine is a glycoprotein that influences the proliferation, survival, maturation and functional activation of cells from the neutrophilic granulocyte lineage. Its main clinical application is to reduce the time of neutropenia (Creaet et al. *Crit Rev Oncol Hematol*, 72:21-44, 2009). Our group reported the production of two transgenic goats containing hG-CSF fused to goat  $\alpha$ -S1 casein promoter (Freitas et al. *Small Rum Res*, 105:105-113, 2012). Later, we have demonstrated that the female founder successfully expressed the recombinant protein in her milk (Moura et al. *Animiotechnol*, 24:10-14, 2013). The aim of this study was: a) to verify the hG-CSF expression during lactation of F1 goats; b) to investigated the ectopic expression by ELISA and by qRT-PCR. It was used transgenic (n = 6) and non-transgenic (n = 6) goats that received a hormonal treatment for induction of lactation. Despite the lower milk production, transgenic females presented asimilar milk composition (fat, protein and lactose) when compared to non-transgenic (P > 0.05). The mean concentration ( $\pm$ SD) of recombinant hG-CSF in milk during lactation was  $360 \pm 178$   $\mu$ g/ml. All clinical parameters, as well as kidney and liver function, indicated that F1 transgenic goats were healthy. Additionally, no ectopic hG-CSF expression was detected. Thus, F1 hG-CSF-transgenic goats can express there combinant protein in milk at quantities compatible with their use as bioreactors in a commercial-scale protein-production program.



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## **Perspectives and cloning outcome at cenatte embriões/semex**

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**Keywords:** calves, somatic cell nuclear transfer, zebu cattle.

Nuclear transfer is a biotechnology with emerging commercial applications and its efficiency has been improved despite the low productivity. The low rates of healthy clones are attributed to high incidences of placental abnormalities leading embryonic and fetal losses (Hossain et al. BMC Genomics, 43:1-15, 2014). Aiming to demonstrate the efficiency of CENATTE Embriões/SEMEX team, we monitored 946 cloned embryo transfers produced from 22 cell donor animals (20 female and 1 male) from October 2009 to August 2013 (breeds: Gir, Brahman, Guzerá, Nelore and Tabapuã). The fibroblasts were obtained by explants from caudal fold and the cloning technique was performed with micromanipulators according to Campbell et al. (Cloning and Stem Cells, 3:201-208, 2001). Efficiency rates were evaluated by pregnancy rate, gestational losses and production of healthy clones within 3 months after birth. From 946 embryo transfers, 42.3% (400/946) recipients were pregnant at 30 days and 22.3% (211/946) at 60 days post embryo-transfer. The gestational losses reached 47.2% (189/400) between 30 and 60 days and 78.5% (314/400) between 30 days to term. The birth rate was 8.9% (84/946) with 78 clones alive and six stillborn. The pregnancy and gestational losses data are similar to those presented by Heyman et al. (Biol Reprod, 66:6-13, 2002), which obtained 33.5% (45/133) of pregnancy at 35 days, 57.7% (26/45) gestational losses between 35 and 70 days and 80.0% (36/45) between 35 days and parturition. We found a postnatal survival rate of 73.1% (57/78). However, 30.8% (24/78) of calves had some form of physical defect. Considering the total of 946 ETs, we delivered to the farmers one male and 32 female healthy clones at third month after birth, representing an efficiency of 3.5% (33/946) for the entire process of commercial bovine cloning. This rate is similar to literature data that shows that less than 5% of embryos clones become healthy calves (Palmieri et al. Vet Pathol, 45:865-880, 2008). Considering the different breeds used by our company, the cloning efficiency was 4.6, 4.4, 1.2, 2.4 and 9.4% to Gir, Brahman, Guzerá, Nelore and Tabapuã, respectively. The cloning efficiency variation among cell donor animals was 0.87 to 12.5%. However, 31.8% (7/22) of the cell donors never produced healthy calves. To enhance the viability of clone calves is necessary to improve the techniques of nuclear transfer and neonatal care as well. This would allow a greater productivity gain of the entire commercial cloning technique in bovine.