Viral vectors with applications in transgenesis: complementation between adenoviral an lentiviral vectors

J.R. Toledo[1], O. Sáchez, R. Montesino, M. Barrera, J.A. Cremata* (*in memory of*).

Biotech and Pharmaceutical Laboratories, Department of Pathophysiology, School of Biological Sciences, University of Concepción, Chile. [1]E-mail: jotoledo@udec.cl.

Keywords: milk expression, recombinant proteins, transgénesis, viral vectors.

Viral vectors are a useful tool for genetic transformation in embryos and cells. Unlike conventional systems of transfection, microinjection or nuclear transfer of DNA, those involve a random insertion of genetic material, the viral vectors use the native system of the infective virus to internalize host cell and transfer its genetic material to the expression environment in the nucleus, increasing the transgenes integration and expression. Our investigation group has developed an application of combining adenoviral with lentiviral vectors to induce the expression of the transgenes in a stable mammary gland, as a target organ for the production of recombinant proteins with pharmaceutical interest. Adenoviral vectors have the advantage of being produced in high titres, and massively transform cells and tissues, quiescent as well as in division. These characteristics have allowed us to produce complex and glycosylated recombinant proteins in the ranges of g/L in mammary glands and cell cultures, obtaining productive levels at least three days post-transformation. This procedure allowed to evaluate the structural characteristics, glycosylation patterns, biological activity and immunogenicity from our transgenes, (such as hGH, hEPO, hFVIII and viral antigens like E2 protein from CSFv, VP60 from HRDv and Alv), expressed in the mammary gland without the obligation of generating stable transgenic animals. With this functional evaluation we can selected the feasible transgenes to produce in milk. Thus, transgenic animals through injection of lentiviral vectors into the perivitelline space were generated with a high efficiency; which mediated the increase in the transgene integration. They significantly raise the efficiency of generation of transgenic animals, obtaining over 80% of animals carrying the transgene. Complementing both methods allows in a short period of time the selection of feasible transgenes for mammary gland expression in transgenic ruminants, which will be used as bioreactors for valuable recombinant proteins.

Recent advances in mammals cloning

D. Salamone, A. Gambini, R. Bevacqua, L. Moro, N. Canel, M.I. Hiriart, C. Buemo

Laboratorio de Biotecnología Animal, Departamento de Producción Animal, Facultad de Agronomía, Universidad de Buenos Aires, Argentina.

Keywords: cloning, nuclear transfer, trophoblastic complementation.

The present review briefly summarizes our experience with somatic cell nuclear transfer (SCNT) to reproduce elite animal, produce transgenic animal and for to preserve endangerous species. We have been using 3 different technique of SCNT in bovine, caprine, equine, swine and feline. High efficiencies in enucleation of recipient oocytes have been achieved using DNA specific vital dyes to visualize chromatin using zona pellucida (ZP) free or ZP included oocyte. Fusion of the donor cell was done by electric pulse with the recipient oocytes were use in both cases. In ZP included enucleated oocytes nucleus can also be injected opening the possibility to use death cell. A problem with existing nuclear transfer systems is that the survival of cloned embryos and fetuses is low. We assessed a new alternative cloning technique, previously described in the bovine, which consists of aggregating zone free genetically identical cloned embryos as a strategy to improve in vitro and in vivo embryo development in the equine. Embryo aggregation improved the quality of in vitro equine cloned embryos at day 7, and pregnancy rates were higher. Different strategies were used successfully multiplied transgenic embryos embryos including the separation of transgenic blastomeres followed by the aggregation of two-cell fused embryos or by the asynchronous younger blastomere. This trophoblastic complementation technic can also be used to produce pregnancies from induced pluripotencial stem cells (IPS) from animals with high genetic value or transgenic cell lines. Another technique that was developed was gamete cloning. We demonstrated that a sperm and oocyte can be efficiently cloned. This approach shows great potential because it allows for determination of the sex of the sperm nucleus prior to fertilization. It is also possible to clone previously transfected oocytes followed by the reconstruction of biparental bovine embryos to generate homogeneous transgene-expressing embryos. This opens the possibility for sperm or oocyte genome cloning by multiplying the gamete in a haploid line. This would have the potential to generate an unlimited number of biparental embryos by combining these haploid cells with haploid hemizygotes of the opposite sex. We have developed a new method to replicate somatic micronuclei, by using the oocyte replication machinery. It could be a useful tool to transfer a small number of specific chromosomes and to target transgenesis to a reduced area of the genome. All these approach has enormous potential for use in livestock production. One of the main applications is introducing genes to modify the genome for biomedicine or agriculture or to multiply genetic elite or endangerous willife animals.

Transgenic animals in Agriculture

J.D. Murray

Department of Animal Science and Department of Population Health and Reproduction University of California, Davis CA 95616 USA.

Keywords: agriculture, GE, GMO, livestock, transgene.

As soon as the first publications came out reporting the effects of human growth hormone in transgenic mice speculation began about how genetic engineering could be applied to improve livestock for use in agriculture. However, 30 years later this promise has yet to be realized as there are no GE animals approved for food consumption anywhere in the world. The reasons for this will be discussed and included a number of possible explanations. Initial work mostly concentrated on the production of animals (sheep and swine) with deregulated growth hormone (GH) genes. While a variety of GH transgenic lines of livestock were successfully produced, most had compromised health and were not suitable for further development. However, there were a number of lines of transgenic farm animals and fish produced during the 1980s and 90s that did not have health issues and were potentially useful genotypes for use in production agriculture. To date none of these lines have yet been approved for human consumption and thus have not made it into production. Many of these lines have been placed in frozen storage or have been lost and would need to be re-developed. The reasons for this are varied and include simply retirement of the developer, through to insufficient funds, pressure from anti-biotechnology activists, lack of a regulatory process, public opposition, or lack of a perceived need by industry. The combination of these factors has led to a virtual cessation of research and development of transgenic animals for use in agriculture in Europe and Australia, early leaders in developing this technology and the emergence of programs in places like Brazil, China and Argentina.

The dark side of producing recombinant proteins in the milk of transgenic animals

H. Baldassarre

McGill University, Ste. Anne de Bellevue, Quebec, Canada.

Keywords: endoplasmic reticulum stress, milk composition, tight junction integrity, transgenic goat.

Genetically modified mammalian cells, mainly Chinese Hamster Ovary (CHO) cells, have been successfully used for over 2 decades in the production of biopharmaceuticals that are too complex for expression in bacteria and yeast. These cells are better equipped for complex protein synthesis, so this system often provides recombinant proteins with adequate post-translational modifications, although some of the sugars are not completely added. Its main drawback is the need for large capital investment, high operating costs and relatively low production levels, resulting in the incapacity to produce more than a few kilograms of protein per year using this platform. Given these limitations, the transgenic animal platform, in which recombinant proteins are expressed in the mammary gland of transgenic animals and purified from their milk, emerged in the 90's as a promising method for the production of valuable therapeutic proteins that cannot be adequately expressed in other systems. The system offers the outstanding protein synthesis capacity of the mammary gland, rather low capital investments (animals and barns), low operating costs and virtually unlimited capacity to scale-up by simply breeding more animals. While these statements stand true, these economic advantages only impact on the upstream aspect of pharmaceutical production which accounts only for <10% of the cost of goods. Conversely, the downstream processing of milk-expressed biopharmaceuticals is significantly more expensive because it starts from a rather complex raw material (milk) and is produced in a system that is not "friendly" for the regulatory eye. Another negative aspect that is generally overlooked is the fact that the expression of large quantities of recombinant proteins in the mammary gland can result in phenotypes with compromised lactation physiology. These negative consequences have been vaguely described in the literature and, in most cases; they have not been studied in depth with view to understanding the mechanisms of mammary disruption by transgene expression. In recent years, we explored the lactation performance and milk composition of transgenic goats expressing recombinant proteins in the milk, using a transgenic herd of goats expressing recombinant human butyrylcholinesterase. Our findings showed that transgenic lactations were characterized by a slow/delayed start of milk production, a relatively normal milk volume at peak and a premature shutdown of milk production compared to controls. These compromised productivities were associated with a disrupted lipid secretion at the level of the secretory epithelium, and a dramatic raise in the presence of phagocytes in milk that was not associated with mammary infection. Milk composition studies indicated that transgenic goats produce lower quantities of caseins and short chain fatty acids. Through determination of serum album presence and Na:K ratio in milk we established the development of permeable tight junctions as an apparent mechanism of lactation disruption in the transgenic animals. Delayed expression of α-lactalbumin was proposed as a major determinant of the delayed lactogenesis observed in the transgenic goats. Finally, immunohistochemistry studies revealed increased expression of ER-stress signalling molecules (ATF6 and Caspase 12) in transgenic goats. In conclusion, several mechanisms of mammary disruption in transgenic goats were established and this knowledge was later applied to the development of treatments that can successfully improve productivity and recombinant protein output in transgenic goats.

Mammary-specific transgenes: Past, present and future

Elizabeth A. Maga

University of California, Davis, USA.

Keywords: livestock, mammary gland, transgenesis.

The goal of mammary-specific transgenesis is to produce a specific protein in the milk of an animal either for purification for use as a pharmaceutical or to modify the functionality or nutritional content of the milk itself. The first mammary-specific transgenic animals were generated 25 years ago, not long after the first report of transgenesis in mammals. The expression of the transgene can be restricted to the mammary gland using a promoter and regulatory elements of a milk protein gene. There are six main milk protein gene products that are made only in the mammary gland and only during lactation including four caseins (α_{s1}, α_{s2}, β and κ) and two whey proteins (β-lactoglobulin and β-lactalbumin). Original mammary-specific vectors included varying promoter regions of several of these genes including sheep β-lactoglobulin, bovine α_{s1} and β-casein, and the mouse whey acidic protein (WAP). Work ensued characterizing important regions of these genes required to direct efficient mammary-specific expression of the recombinant product. A large bovine α_{s1}-casein expression cassette and a goat β-casein cassette, including β-globin insulators to eliminate position effects, were frequently used for gene addition via pronuclear microinjection as they direct high levels of expression specifically in the mammary gland. These types of transgenes have also been used in the presence of RecA, a bacterial-derived protein responsible for homologous recombination, in attempts to increase transgene integration efficiency. The genome sequence of many livestock species is now available and will allow for the generation of mammary-specific transgenic animals with specific modifications made via gene targeting and subsequent somatic cell nuclear transfer. Lentiviral vectors are also being developed to allow for the more efficient generation of mammary-specific transgenic animals. In summary, standard mammary-specific expression cassettes can be used to routinely generate transgenic livestock that express the transgene in their milk and newer methods are being developed to allow for more precise modifications of milk.

Production and characterization of transgenic founder goats for hG-CSF for further transgenic herd formation

V.J.F. Freitas[1], L.M. Melo, A.F. Pereira, D.I.A. Teixeira, R.I.T.P. Batista

Laboratório de Fisiologia e Controle da Reprodução, Universidade Estadual do Ceara (UECE), Brasil.
[1]E-mail: vicente.freitas@uece.br

Keywords: Goat, hG-CSF, pronuclear microinjection, transgenesis.

The production of recombinant human proteins with pharmaceutical uses in the milk of farm animals can overcome the limitations facing traditional recombinant pharmaceutical protein production systems. The mammary gland is the preferred protein production site, due to the quantities of protein that can be produced. Once a founder that has a stably integrated transgene and is correctly expressing the gene has been produced, propagation of a transgenic herd may be initiated. In this study, we describe the production of transgenic founder goats for human Granulocyte Colony Stimulating Factor (hG-CSF), a partial description of the phenotypic characteristics of the founders, including milk hG-CSF production, and the propagation of a transgenic herd. After microinjection and embryo transfer, 12 recipients became pregnant and produced 18 kids, including one male (10M) and one female (12F) that carried the transgene. The two transgenic founders remained healthy throughout the experimental period and thereafter. At 280 days of age, both the male and female were sexually active. Despite of the neutrophil oscillations, hG-CSF was not detected in serum samples using high sensitivity ELISA assay (from 1 pg/ml). Additionally, the hematological findings were not accompanied by changes in other cell counts, biochemistry serum measurements or clinical parameters. Both the transgenic and the non-transgenic females produced milk after the hormonal induction of lactation. The average concentration of hG-CSF produced by the transgenic female was 620.9 ± 179.9 μg/ml of milk. To determine the biological activity of hG-CSF in milk, we examined its effect on precursor cells of human umbilical cord blood. Addition of the milk of the transgenic goat containing 50 ng/ml of hG-CSF produced a significant increase of the number of the colonies when compared to negative control (12.2 ± 4.2 versus 1.2 ± 0.9 CFU, $P < 0.01$). A total of nine transgenic (four females and five males) and ten non-transgenic (five females and five males) kids have been produced by founders. In both 10M and 12F lines, the transgene segregated equally among the male and female offspring. In summary, we produced two transgenic goats with a stably integrated hG-CSF gene that were capable of secreting recombinant hG-CSF from the lactating mammary gland without causing any harm to the animals' health. Additionally, transgenic goats are true founders as they were proven to be fertile and capable of transmitting the hG-CSF gene to first generation progeny. Additional investigations regarding the phenotypic and genotypic characteristics of the 10M and 12F lines are warranted. We believe that the experiments presented here provide an efficient strategy that may be useful for the industrial production of recombinant hG- CSF protein using transgenic goats.

Acknowledgments: The authors thank the staff of the Laboratório de Fisiologia e Controle da Reprodução for technical assistance and animal care. This work was financed by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, Brazil), Financiadora de Estudos e Projetos (FINEP, Rio de Janeiro, Brazil).

Trichostatin on production of genetically modified bovine embryos by somatic cell nuclear transfer

L.S.A. Camargo[1], C.C.R. Quintão, L.T. Iguma, L.G.B. Siqueira, B.C. Carvalho, J.H.M. Viana

Embrapa Gado de Leite, Juiz de Fora, MG. [1]E-mail: camargo@cnpgl.embrapa.br

Keywords: cattle, cloning, transgenesis.

Somatic cell nuclear transfer (SCNT) has been described as an alternative to generate genetically modified livestock animals once this method allows that cells be cultured for several passages, a requirement to establish lineages of genetically modified cells, can be used as nuclear donors. Nevertheless, cellular and molecular mechanisms involved on nuclear reprogramming are still unknown, reflecting in the low efficiency of SCNT in producing viable offspring. Moreover, long-term cell culture, required for establishment of genetically modified cell lineages, induces senescence and can impair nuclear reprogramming (Banito *et al*., 2009. Genes Dev, 23:2134-2139; Tat *et al*., 2011. Cell Reprogram, 13:331-344) and reduce embryo quality (Jang *et al*. 2004. Theriogenology, 62:512-521), despite viable bovine clones have already been born from cell cultured for several passages (Kubota *et al*. 2000. Proc Nat Acad Sci, 97:990-995). The modulation of nuclear reprogramming by chemical agents, like inhibitors of histone deacetylase and methylation, has been studied and can be useful for donor cells difficult to reprogram. One of histone deacetylase inhibitors is the trichostatin A, but studies with it produced controversial results. Some studies showed that exposing zygotes clones to 50 nM Trichostatin can increase bovine blastocyst production (Lee *et al*., 2011. J Reprod Dev, 57:34-42; Sawai *et al*., 2012. J Reprod Dev, in press), but such results were not repeated by others, which also did not find improvement on pregnancy and birth rates (Cui *et al*., 2011. Cell Reprogram, 13:179-189; Sangalli *et al*., 2012. Cell Reprogram 14:1-13). In previous study, we also observed that trichostatin treatment did not increase blastoscyst rate but reduced the index of apoptotic cells and end out with the birth of one animal (Camargo *et al*., 2011. Acta Sci Vet, 39(supl):S442); however, alteration on expression of genes important for development, like IGF2r and HMGN1, were found in blastocysts, suggesting that the reprogramming was not completely successful (Camargo *et al*., 2012. Reprod Fertil Dev, 24:121-122). Recently, we observed that the trichostatin treatment of zygotes reconstructed with genetically modified somatic cells after long-term culture (12 passages) increased the blastocyst rate (10.3±3.6% vs 26.7±3.8% for control [untreated zygotes] and zygotes treated with 50 nM trichostatin, respectively; unpublished data), contrasting with our previous results with zygotes reconstructed with non-transgenic cells with low-passage number (4-6 passages). The trichostatin treatment did not interfere on expression of reporter gene (GFP). This positive result with genetically modified donor cells may be due to the trichostatin effect on zygote reconstructed with cells cultured by long periods, as required for the establishment of the transgenic lineage. Therefore, the effect of trichostatin may be effective in zygotes clones reconstructed with cells difficult to reprogram, like those transgenic ones cultured for long-term and possibly close to senescence. Nevertheless, it is necessary to evaluate whether the increase on blastocyst production reflects on improvement of quality and pregnancy rate of transgenic clone embryos.

Supported by FAPEMIG and CNPq.

Are stem cells from farm animals a reliable source for nuclear transfer?

F.O. Castro, L. Rodríguez

Department of Animal Science. Faculty of Veterinary Sciences. Universidad de Concepción, Chillán, Chile.

Keywords: bovine, iPS cells, mesenchymal stem cells, nuclear transfer.

There is not a clearly defined, unique source of appropriate cells for nucleus transfer in farm animals. Enormous efforts had been conducted to predict the outcome of cloning based on cell markers expressed in the donor cells; however these efforts had rendered relatively ineffective so far, except for limited accomplishments in identifying pluripotent markers in donor cells, which in turn showed a correlation with better early embryonic development in cattle (unpublished results from our group and other cites). Embryonic, induced pluripotent or adult cells constitute promising alternatives for more efficient cloning procedures, but again no clear advantages of such cells have been shown or published. Although cloned mice derived from iPSc cells had been produced, same results are still missing for farm animals. Although there are a few papers communicating improved embryonic development after using stem cells in cloning, for instances in goats (Dutta *et al*. 2011. Theriogenology, 15:851-863) or pigs (Shin *et al*. 2012. Zygote, 20:9-15), no conclusive evidences of improved embryo development can be withdrawn so far neither from reviewed literature nor from our own data. The aim of our research is to isolate stem cell lines that can be useful for bovine cloning; this includes the search for induced pluripotent stem cells and also for embryonic-like and adult cells. In this presentation we will review the state of the art of animal cloning using stem cells and will show the progress of our research group in isolating stem cells from cattle. In conclusion, more research is needed in order to generate efficiently cloned farm animals using stem cells as donors. This obviously relies also in the improvement of methods to isolate, expand and characterize these cells from economically relevant animal species.

Selection of nucleus donor cell for somatic cloning

L. Rodríguez-Alvarez, F.O. Castro

Department of Animal Science. Faculty of Veterinary Sciences. Universidad de Concepción, Avenida Vicente Méndez 595, Chillán, Chile.

Keywords: donor cell, gene expression, nuclear transfer, reprogramming.

Nuclear transfer (NT) is a complex process in which a differentiated cell is reprogrammed to induce embryo development. The efficiency of nucleus reprogramming depends on different factors, but the characteristics of the donor cell are one of the most important. In this regard, different aspects have been studied including cell type origins, animal donor age and cell cycle stage. Certain cell types are more suitable to reprogramming, independently of their origin, but depending on their epigenetic status, thus opening the way to the use of epigenetic modifiers as an approach to increase cloning efficiency. The gene expression profile of the donor cell will impinge upon gene expression patterns of the derived embryos and on their developmental potential. It is known that the expression of a specific gene in the donor cell facilitates the expression of that gene in the resulting embryo after NT and vice versa. For example, mouse blastocysts produced by NT from ES cells that expressed OCT4 were able to rescue OCT4 expression in 100% of the cases. In bovine, we found that the expression level of OCT4 in the donor cells positively correlated with both embryo development capability and quality. Also, a higher expression level of OCT4 in the donor cell increased the expression levels of Oct4 and Sox2 in grade-I cloned blastocysts. However, the variability of expression of those genes among embryos was also high, using the same cell line. This probably indicates that the oocyte is unable to correct for high Oct4 levels. This can be an explanation as to why using stem, ipS or stem cell-like cells as source of nuclei does not improve the actual cloning efficiency. In conclusion, much is still required to investigate in order to select cell lines more amenable for nucleus reprogramming and embryo ability to produce healthy offspring.

Post-thaw function of cryopreserved secondary follicles from ovarian tissue in the rhesus macaque

C.C.D. Almeida-Francia[1]*, J.R. Campos[2], A.Y. Ting[3], R.R. Yeoman[4], M.S. Lawson[5], M.B. Zelinski[6]

[1]Department of Anatomy, Institute of Biosciences, Univ Estadual Paulista, Botucatu, SP, BRASIL; [2]Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, BRASIL; [3,4,5,6]ONPRC, Oregon Health & Science University, Beaverton, USA.

Keywords: cryopreservation; secondary follicles; Rhesus macaque ovary.

Cancer treatments can deplete follicles in the ovary leading to premature ovarian failure and infertility. Cryopreservation of isolated secondary follicles followed by encapsulated 3-dimensional (3D) culture to produce a fertilizable oocyte is being investigated as a potential option to restore fertility in female cancer patients who are pre-pubertal or require immediate cancer therapy. Following cryopreservation, fertility may be restored after *in vitro* follicle maturation. The function of secondary follicles after cryopreservation and thaw was compared with fresh follicles during 3D culture. Ovaries were obtained from adult rhesus macaques (n=4) at day 1-4 of early follicular phase of the menstrual cycle. The cortex was cut into 1x1x0.5mm^3 pieces, and secondary follicles were isolated mechanically without enzyme digestion. For cryopreservation, individual follicles were vitrified in solutions containing glycerol (GLY), ethylene glycol (EG) plus PXZ polymers (polyvinyl pyrrolodine, X-1000 polyvinyl alcohol and Z-1000 polyglycerol). Fresh and vitrified-thawed secondary follicles were encapsulated in 0.25% alginate, transferred to individual wells of a 48-well plate containing 300µl of alpha minimum essential medium culture media containing glucose, FSH, serum protein supplement, fetuin, insulin, transferrin, selenium, ascorbic acid phosphate and cultured at 37^o C in 5% O_2 (in 6% CO_2/89% N_2) for 5 weeks. Follicle health and diameter were assessed weekly by microscopy. Five-week survival rate for follicles from vitrified tissue (35%) was reduced relative to those from the fresh tissue (79%). Antrum formation was observed by weeks 2-4 in 75% of fresh follicles that survived 5 weeks of culture, but was diminished in the vitrification group (17%) in comparison to the fresh. Follicle diameters were similar in fresh and vitrified groups on the day of isolation. Follicles from fresh tissue showed increased diameters during week 3 and continued to grow throughout culture. Follicles previously vitrified that formed an antrum also grew in culture, but showed delayed and reduced growth in week 5 when compared with the fresh. Isolation of follicles for subsequent cryopreservation is a relatively new approach for fertility preservation. Our results in the nonhuman primate suggest that vitrification with GLY, EG and PXZ polymers in a closed system has potential as a method for cryopreservation of isolated secondary follicles. Whether this technique can yield oocytes capable of fertilization leading to live offspring remains to be determined. Vitrification of secondary follicles would provide an option for fertility preservation in patients where ovarian tissue transplantation poses a risk transmitting of malignant cells.

Grants acknowledgements: Oncofertility Consortium [NIH UL1 RR024926, R01AHD058293, PL1 EB008542], SCCPIR (U54 HD018185), P51RR000163.