Transgenic bovine as bioreactors: Challenges and perspectives

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ABSTRACT
The use of recombinant proteins has increased in diverse commercial sectors. Various systems for protein production have been used for the optimization of production and functional protein expression. The mammary gland is considered to be a very interesting system for the production of recombinant proteins due to its high level of expression and its ability to perform post-translational modifications. Cows produce large quantities of milk over a long period of lactation, and therefore this species is an important candidate for recombinant protein expression in milk. However, transgenic cows are more difficult to generate due to the inefficiency of transgenic methodologies, the long periods for transgene detection, recombinant protein expression and the fact that only a single calf is obtained at the end of each pregnancy. An increase in efficiency for transgenic methodologies for cattle is a big challenge to overcome. Promising methodologies have been proposed that can help to overcome this obstacle, enabling the use of transgenic cattle as bioreactors for protein production in milk for industry.

Introduction
Recombinant proteins have been used in several industrial sectors, primarily the pharmaceutical, food, cellulose, detergent, textile and biofuels sectors, among others. The selection of the system for recombinant protein production is based on the intrinsic characteristics of the native protein, and therefore, post-translational modifications must be considered. Most of the proteins used as therapeutic agents in human diseases are processed after translation to acquire their functional conformation. Mammalian cells are able to perform post-translational modifications similarly to the human cells and are used to produce therapeutic proteins. However, mammalian cell culture requires specific conditions and culture medium that typically leads to a high cost of protein production. This results in therapeutics that become very expensive for the patients. Alternatively, the mammary gland has been considered an important system for the production of recombinant proteins, which has the great advantage of high protein production in a native conformation at a low cost. The generation of transgenic animals with the goal of producing recombinant proteins in milk has been accomplished in a number of different livestock species including rabbits, pigs, goats, sheep and cattle.

The choice of the specific animal species for recombinant protein production in the milk is influenced by the market demand, the volume of milk produced per lactation cycle and the reproductive rate. Dairy cows produce 1 to 14 g/L of heterologous protein in milk daily for a 305 day lactation cycle each year. In contrast with mammalian cell culture, recombinant protein production has increased, mainly due to media improvements, with a yield of more than 10 g/L protein in 10 to 12 d in CHO (Chinese hamster ovary) cell large-scale, serum-free, suspension batch-fed cultures. The total yield of recombinant protein production is higher in milk than in mammalian cell culture, and no specific conditions, media, or cells are required. The recombinant protein can be harvested several times per day at a large scale (>50 L) and is readily purified from milk. However, recombinant protein production is initially limited due to the long
interval from birth to the first lactation in the domestic animals. Further, the discontinuous lactation cycle and the substantial investments in time and material to produce transgenic animals must be considered as a limiting factor.6

Transgenic cattle are difficult to generate principally due to the low efficiencies of transgenesis techniques and by the long time period required for evaluation of the transgene and the production of the recombinant protein. Different methodologies have been considered promising for the generation of transgenic cattle; however, somatic cell nuclear transfer (SCNT) is the principal tool used due to the high rates of cloning results in a low transgenic founder efficiency. The generation of transgenic cattle is also very expensive, as significant infrastructure must be established and the efficiency of transgenic production is low. The infrastructure required includes the training or hiring of specialized technicians, a large number of animals for use as embryo recipients, confinement and feeding space for recipient animals, suitable facilities for the birth and care of the transgenic calves and an appropriate confinement areas for transgenic animals.

Despite these limitations, 2 biopharmaceuticals produced in the mammary gland of transgenic animals have been released and are commercially available for treating human disease: antithrombin (ATryn*), produced by GTC Biotherapeutics UK Limited (recently acquired by LFB Biotechnologies), and C1-esterase inhibitor (Ruconest*), produced by the Pharming Group, NV. Other biopharmaceutical companies are working to produce diverse biopharmaceuticals in the milk of transgenic animals.7 If we can increase the efficiency of the generation of transgenic cattle, this system will likely become one of the most attractive methods for recombinant protein production. This will also make possible the use of various proteins in various industrial sectors beyond the current pharmaceutical usage.

Vectors used in the generation of transgenic animals

The expression of recombinant proteins in the milk of transgenic animals is driven by the promoter regions from the genes involved in the generation of specific milk proteins, such as caseins (α, β, γ and κ), β-lactoglobulin and α-lactalbumin. The promoter region localized at the 5'-UTR (untranslated region) of the gene of interest, including tissue-specific enhancers for the mammary gland and the first non-coding exons and introns, is variable in size and dependent of the promoter used. Bovine α-lactalbumin and β-lactoglobulin promoters have been used with approximately 2.0 kb and 2.8 kb gene sizes, respectively,8,9 while casein promoters (αS1, αS2 and β) have generally been used for genes from 3.1 kb to 14.2 kb in length.10,11 Other regulatory elements, such as insulators, have been added upstream of the 5'-UTR in the vector construction to ensure high-level and/or position-independent expression of the transgene. One example of this is the commercial pBC1 vector that includes the chicken β-globulin insulator (Invitrogen – Thermo Fisher Scientific). The first non-coding exons and introns in the 3'-region of the 5'-UTR are important in that they position the mRNA in ribosomes to start their translation and/or contain regulatory elements that can enhance gene transcription. Generally, these non-coding exons and introns are derived from milk protein genes but can be derived from the structural gene used in the transgene construction.

Typically, the gene of interest is inserted in the vector downstream from the 5'-UTR either as a cDNA sequence, a complete gene sequence (containing exons and introns) or as a mini-gene. For recombinant proteins to be secreted in milk, it is essential that the signal peptide sequence is included, which is usually from the transgene used. During vector construction, an alternative vector is one where the promoter region from a milk protein gene is cloned, including the signal peptide sequence from that milk protein gene. This vector can be used for the production of recombinant proteins in milk that are not naturally secreted.

The 3'-UTR is inserted downstream of the transgene and can be from the same milk protein gene used in the 5'-UTR, from the structural gene used in the transgene or from a different gene. This region ensures the efficient transcriptional termination and the formation of a stable mRNA encoding the target protein. In some cases, this region can be as long as 7.1 kb, as in the pBC1 vector (Invitrogen), which includes introns, exons and the polyadenylation signal from goat β-casein. The 3'-UTR may contain regulatory elements that improve the transcription. The polyadenylation signal from bovine growth hormone, which is approximately 1 kb, is a 3'-UTR typically inserted for vector expression.10
Three major vectors classes are used for the generation of transgenic cattle: bacterial artificial chromosomes (BAC), plasmids and lentiviruses. These vectors are chosen based on the length of the transgene. BAC vectors, which can hold DNA sequences up to 300 kb, is the primary vector used when long sequences, such as complete genes and/or long 5′ and 3′-UTRs, must be inserted into host genomes. Transgenic cattle have been generated using this vector. Plasmid vectors are extrachromosomal, self-replicating DNA molecules. Many of the plasmid vectors are only 3 to 5 kb in length. Plasmids permit the insertion of a DNA sequence up to 20 kb and have been used for the generation of transgenic animals. The commercial pBC1 vector with 21.6 kb of DNA has been used directly or modified to generate transgenic cattle. The lentiviral vectors have a restricted capacity to insert DNA, and the ideal size of genetic material to be packaged into lentiviral particles is approximately 10 kb in length. It is possible to produce lentiviruses with proviral lengths in excess of 18 kb, but the viral titers decrease semi-logarithmically with increases in vector length. This reduction in titer appears to occur at the level of viral encapsulation and/or by limitations in the nuclear export of the proviral RNA rather than at the level of packaging into the lentiviral particles.

During the construction of the lentiviral vectors, it is essential to limit the length of the vector. The viral titer can be improved using cDNA and not complete gene sequences, as well as including a shorter mammary gland-specific promoter region and polyadenylation signal, such as the α-lactalbumin promoter and the bovine growth hormone poly-A sequences. These modifications can improve the viral titer from lentiviral vectors. The essential viral elements present in the lentiviral vector, such as the LTR, the packaging signal, a central DNA flap and a WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), are approximately 1.5 kb in size; therefore, the transgene and the 5′ and 3′-UTRs together have to be smaller than approximately 8.5 kb.

The use of lentiviral vectors to express recombinant protein in milk was used successfully in transgenic mice, while in cows it has been used to express green fluorescent protein (GFP). In contrast to the plasmid vectors, there are no commercial lentiviral vectors available for expressing recombinant proteins in milk, although some authors have modified commercial lentiviral vectors for this purpose (Fig. 1). Efforts to generate transgenic cattle for the production of recombinant proteins in milk using lentiviral vectors have been successful. It has been proposed that lentiviral

![Figure 1. Lentiviral vector construct for targeting recombinant proteins into milk (for details see reference18).](image-url)
vectors have advantages over other vectors mainly because of the high percentage of transgene expression in the founders. This high expression occurs because the integration of the lentivirus occurs preferentially in active transcriptional units in the host genome.20

The use of plasmid vectors based on transposons system has emerged as an attractive alternative for generating transgenic animals. Sleeping Beauty, piggy-Bac and Tol2 transposons have been developed as gene transfer methods for vertebrate genetics, and commercial vectors are available (Addgene). The gene of interest is cloned between the transposon inverted terminal repeats (ITRs) that carry the binding sites for the transposase enzyme. The gene is mobilized by supplementing the transposase enzyme from a second expression plasmid or by the synthetic transposase mRNA. This system has the ability to transpose up to 18 kb transgenes, but the transposition efficiency significantly decreases with increasing DNA cargo sizes and appears to be dependent on the cell type used.21 Transposon plasmids have also been developed for use in transgenic procedures,22 and the generation of transgenic pigs and rabbits using this system was successful.23,24

**Evaluation of the vectors constructed**

Prior to the generation of transgenic animals as bioreactors, it is important to evaluate the vector construction of the transgene for functionality of the promoter, its ability to respond to hormonal induction and its capacity to express the recombinant protein of interest. The evaluation of recombinant protein production in milk of transgenic cattle involves a long interval from birth until the first lactation cycle, thus it is essential to estimate the efficiency of the vector in the production of the recombinant protein. The production costs for transgenic cattle can amount to $300,000 to $500,000 per animal if a pronuclear DNA microinjection technique is used or a few thousand dollars if lentiviral gene transfer into the perivitelline space of bovine oocytes is used.16 The use of time and resources must be optimized in the generation of transgenic cattle, and vector expression evaluation is an initial important step during this process.

The production of transgenic mice has been successful using different techniques, and these animals are used as the main model of animal transgenesis due to their high reproductive rates and the short time required to achieve puberty, mating and lactation. The first transgenic mice by microinjection of recombinant DNA into the pronuclei of fertilized eggs were generated in 1980,25 and 29 y ago, the β-lactoglobulin gene from sheep was microinjected into fertilized eggs to generate the first expression of recombinant protein in the milk of mice.26 In 1990, transgenic mice were chosen as a model to study recombinant protein expression in milk.27 Although transgenic mice are the best models to evaluate the recombinant protein in milk, their generation depends on specialized technicians for microinjection and reproduction and on vivarium environments with biosafety certifications.

The biology of the mammary gland can be reproduced using primary mammary epithelial cells (MEC) separated from the extracellular matrix, which will form monolayer cell cultures. MEC cultures synthesize and secrete large quantities of milk proteins, and the growth and differentiation stages are controlled by a variety of protein and steroid hormones, in addition to cell-cell and cell-substrate interactions.28 These characteristics make MEC cultures one attractive system to reproduce mammary gland biology. In 1998, Ilan and collaborators proposed that MEC cultures may be an alternative to transgenic mouse models for evaluating the potential of gene constructs to be expressed in the mammary gland of transgenic farm animals.29 Others studies have used MEC cultures to evaluate the expression of the vectors constructed.13,18,19,30 Although transgenic mice may be the best model for transgenic farm animals, MEC culture is a strategy that is easier to execute than the generation of transgenic mice and shows certain advantages, such as a lower cost and an elimination of the necessity for a vivarium to produce and house transgenic mice.

**Strategies for the generation of transgenic cattle - challenges to overcome**

Methodologies used in the production of transgenic cattle have limitations that would impede its use in commercial application. The genetic engineering of livestock was successfully achieved in 1985 by using pronuclear DNA microinjection into fertilized oocytes.31 This technique is very inefficient, with between only 1-5% of large animals born carrying the transgene. Furthermore, it is necessary to transfer large numbers of microinjected embryos to generate a
few transgenic offspring. The use of transgenic technology has been limited in livestock because the gestation time is longer, the maintenance costs are high, principally due to non-transgenic gestation, and the proportions of transgenic animals born that express the recombinant protein are small. Despite these difficulties, pronuclear DNA microinjection was successfully used to generate transgenic cattle.32

Sperm has been used as a vector to introduce exogenous DNA into oocytes because it is a simpler methodology for the generation of transgenic animals. Transgenic pigs were produced using sperm-mediated gene transfer (SMGT) in which the sperm was first incubated with the plasmid DNA.33 However, this method shows a limited integration of the exogenous DNA into the host genome, but variations of the technique have improved the ligation of DNA to sperm, including the use of lentiviruses34. Initially, SMGT was reported to be inefficient with bovine sperm for the production of blastocysts in vitro.35 A modification of this method using spermatogonial stem cells now offers a new method for producing transgenic animals. These cells can be isolated from the testis, modified genetically and transplanted into the seminiferous tubules of males, where they start the production of transgenic sperm to be used in in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) techniques. This methodology has been successfully used in mice,36 but it has not been optimally established for the in vitro culture conditions of large animals.7

The use of embryonic stem cells (ESCs) and embryonic germ cells (EGCs) are promising methodologies for the generation of transgenic large animals. These cells can be cultured and transfected in vitro and are injected into the blastocoele of host blastocysts, which then contribute to the somatic and germ cells of the resulting chimeric animal. When these animals are used in conventional breeding schemes, part of the resulting offspring may be transgenic. Alternatively, modified ESCs have been used in sandwich aggregation with non-viable tetraploid morulae for the generation of transgenic mice entirely derived from ESCs.37 These methodologies have been used in cattle, and chimera animals were generated.38,39 However, the establishment of pluripotent ESCs from bovine embryos has proven unsuccessful, and the generation of transgenic bovine chimeric offspring was only possible using nuclear transplantation, where transgenic embryonic stem (ES)-like cells were produced from modified fetal bovine fibroblasts.40 Transgenic porcine chimeras were generated using primordial germ cell (PGCs)-derived colonies,41 and PGCs can become EGCs by in vitro or in vivo differentiation. Induced pluripotent stem cells (iPSCs) are stem cells that can be generated via cellular reprogramming using gene transduction or recombinant protein treatment of somatic cells. Studies have demonstrated that PGCs obtained by the in vitro differentiation of iPSCs produce functional gametes as well as healthy offspring, reviewed by Imamura et al.42 This is a promising approach, once modified somatic cells can be used to generate iPSCs using a methodology that does not integrate the OCT4, SOX2, c-MYC and KLF4 genes into the genome of the somatic cells previously modified. The iPSCs carrying the transgene of interest can be used to generate PGCs, which can be differentiated into gametes by in vivo or in vitro methods and then injected into the blastocoele of blastocysts for chimera generation.

Viral-mediated gene transfer is another promising methodology for the generation of transgenic cattle. Lentiviruses are natural systems that introduce exogenous DNA into a host genome and are one of the principal systems for transferring DNA in the generation of transgenic animals. Lentiviral transduction is performed by injecting viral particles into the periviteline space of oocytes or zygotes or by removing the zona pellucida and culturing in medium containing the virus. Lentiviral gene transfer into oocytes, using the commercial vector FUGW, was used successfully to produce transgenic cattle.16 This methodology was established more than a decade ago, and although it is regarded as a promising technique for producing transgenic cattle, so far it has not yet been used for the efficient generation of transgenic cattle for the production of biopharmaceuticals in the milk. Efforts along these lines were reported,43 but the increase in the size of the lentiviral vector construct largely decreased the efficiency of lentiviral transduction. Therefore, the efficiency of this methodology is directly related to the transduction capacity of the lentiviral vector constructed.

Similar to lentivirus, DNA transposons integrate into the chromosomes of the host cells. Transposon plasmids were cytoplasmically microinjected to introduce DNA into the fertilized oocytes of pigs, and high rates (40 to 60%) of transgenic founders per animals born were achieved.23 In rabbits, transposon plasmids
were co-injected into the pronuclei of fertilized oocytes along with synthetic transposase mRNA, resulting in efficiencies of transgenesis of 15% or higher.24 Transposon-mediated transgenesis offers comparable efficacies to lentiviral approaches but without the toxicity and biosafety concerns of working with lentiviral vectors.24 The integration by transposons demonstrates more stability and integrity of the transgene compared with the lentivirus system.21 Studies using transposon-mediated transgenesis resulted in a higher efficiency of transgene expression than lentiviruses, and mosaicism in the newborns has not been reported as has been done for lentiviruses.23,24 These features make the transposon system an attractive methodology for the transgenesis in the bovine. However, the efficacy in cattle must be proven principally using promoters driving the recombinant protein expression in milk.

The first transgenic cloned lambs expressing human factor IX were produced in 1997.44 After that time, numerous transgenic animals were produced using SCNT for many scientific proposals, including heterologous protein production. SCNT is the major means for producing transgenic large animals, as 90 to 100% of newborns carry the transgenes.1 However, animal cloning is an inefficient technique due to the low viability of the clones, the high mortality rates during gestation and the fact that most of the living offspring show a high rate of birth defects and pathological features.45 These abnormalities are principally due to aberrant gene expression resulting from epigenetic reprogramming errors in the donor cells.46 In cattle, pregnancy rates at 30 d after the embryo transfer of the cloned embryos is approximately 37%, of which only 9% of the transferred embryos result in calves, and the efficiency ranges are in accordance with the cell lines used.47 However, somatic cells have a limited life span in in vitro culture, and the decrease in developmental rates for modified cells has been attributed to the extended culture periods associated with the transfection and selection procedures before SCNT.48 Studies using different approaches aimed at increasing the efficiency for the generation of viable clones have been performed. The methods investigated include recloning, autologous SCNT, impeding Xist expression from the active X chromosome, treatment with trichostatin A after SCNT and histone deacetylase inhibitors, among others. SCNT has been successfully used for the generation of transgenic cattle for recombinant protein expression in milk aimed at nutritional12,14 and therapeutic50,51 purposes. Effective SCNT can be applied to the production of transgenic animals that could contribute toward the development of sustainable and diversified animal production systems.49 Our research group has used transduction of bovine fibroblasts by lentiviruses, and the modified cells are being used for SCNT for the generation of transgenic cattle. We have generated 2 transgenic calves expressing recombinant human proinsulin and human clotting factor IX (data not published). Expression of the bioactive proteins will be evaluated when these animals undergo lactation.

**Perspectives for transgenic cattle as bioreactors**

The generation of transgenic cattle as bioreactors for recombinant protein production in milk has been focused on nutritional and therapeutics purposes. However, over 500 industrial products are being made using enzymes,52 and the end-use market for industrial enzymes is widespread, with numerous industrial commercial applications.53 The demand for industrial enzymes continuously rises, driven by a growing need to improve product quality, by replacement of the use of chemical pollutants, and by reduction of the intake of raw materials, energy and water, providing benefits to both the environment and industry. However, industrial processes are performed in conditions very different from the cellular process, and the enzymes used must be functional to catalyze these reactions in extreme conditions of pH, temperature, pressure and salinity. Protein engineering methodologies or recombinant DNA technology for the production of enzymes from extremophile microorganisms could yield adequate enzymes for industrial processes. Industrial enzymes are primarily produced by microorganisms, which require high investments in infrastructure and maintenance. This cost of production would be decreased if production is performed by the mammary gland. The construction of expression vectors for targeting recombinant protein into milk, using fusion proteins, such as His-tag, allows for the one-step purification of these proteins. The high protein production combined with lower costs, together with facilitated purification, will make transgenic cattle an attractive system for the production of industrial enzymes. This method would invert the currently used expression systems, in which eukaryotic proteins are expressed in prokaryotic systems, and therefore, some
adjustments such as codon exchanges, must be made. In fact, the use of transgenic cattle as bioreactors at present is still an expensive system for the production of industrial proteins. However, advances in the methodologies involved in the generation of transgenic cattle will allow for the utilization of this efficient system for the production of recombinant proteins.

The modification of milk composition in cattle has had some interest by the dairy industry. Cheese production requires the increased thermal stability that is necessary for cheese production. Therefore, the value of the milk for the production of cheese is increased. Expression of recombinant lysozyme, lactoferrin and lactalbumin in the milk have positive effects on the health of young animals, protecting them against infections and improving the intestinal epithelium structure with positive effects on the intestinal microflora. Expression of lysostaphin in milk increases resistance to staphylococcal infections that lead to mastitis, which decreases milk production. Mastitis is responsible for high loses in milk production, and therefore the control of this infection has a positive impact on dairy farming.

Transgenic cows have been shown to be an excellent system for recombinant protein production that could be used for different commercial applications. The main challenge for the use of transgenic cattle as a system to produce recombinant proteins is increasing the efficiency of generating transgenic cattle. Some promising methodologies discussed here would increase the efficiency, decreasing the cost for the generation of transgenic cattle over the presently used methodologies. Increased efficiency and a decreased cost could allow the use of transgenic cattle as bioreactors for recombinant protein production for diverse industrial applications.

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**References**

[1] Maksimenko OG, Deykin AV, Khodarovich YuM, Georgiev PG. Use of transgenic animals in biotechnology: Prospects and problems. Acta Naturae 2013; 5:53-46; PMID:23556129

[2] Bishop P, Lawson J. Recombinat biologics for treatment of bleeding disorders. Nat Rev Drug Discov 2004; 3:684-94; PMID:15286735; http://dx.doi.org/10.1038/nrd1443

[3] Clark AJ. The mammary gland as a bioreactor: expression processing and production of recombinant proteins. J Mammary Gland Biol Neoplasia 1998; 3:337-50; PMID:10819519; http://dx.doi.org/10.1023/A:1018723712996

[4] Demain AL, Vaishnav P. Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv 2009; 27:297-306; http://dx.doi.org/10.1016/j.biotechadv.2009.01.008

[5] Kim JY, Kim Y-G, Lee GM. CHO cells in biotechnology for production of recombinant protein: current state and further potential. Appl Microbiol Biotechnol 2012; 93:917-30; PMID:22159888; http://dx.doi.org/10.1007/s00253-011-3758-5

[6] Wall RJ, Kerr DE, Bondioli KR. Transgenic dairy cattle: genetic engineering on a large scale. J Dairy Sci 1997; 80:2213-24; PMID:9313167; http://dx.doi.org/10.3168/jds.S0022-0302(97)76170-8

[7] Niemann H, Kind A, Schienieke A. Production of biopharmaceuticals in transgenic animals. In: Kayser O, Warzecha H (eds), Pharmaceutical Biotechnology – Drug discovery and Clinical Applications, 2nd edn. Wiley-Blackwell, Weinheim, Germany 2012; pp. 71-111.

[8] Bleck GT, Write BR, Miller DJ, Wheeler MB. Production of bovine α-lactalbumin in the milk of transgenic pigs. J Anim Sci 1998; 76:3072-3078; PMID:9928612

[9] Hyttinen JM, Korhonen VP, Hiltunen MO, Myohanen S, Janne J. Highlevel expression of bovine beta-lactoglobulin gene in transgenic mice. J Biotechnol 1998; 61:191-198; PMID:9684337; http://dx.doi.org/10.1016/S0168-1656(98)00032-7

[10] Naruse K, Yoo SK, Kim SM, Choi YI, Lee HM, Jin DI. Analysis of tissue-specific expression human type II collagen cDNA driven by different sizes of the upstream region of the β-casein promoter. Biosci Biotechnol Biochem 2006; 70:93-98; PMID:16428825; http://dx.doi.org/10.1271/bbb.70.93

[11] Rijinkels M, Kooiman PM, Platenburg GJ, van Dixhoorn M, Nurjens JH, de Boer HA, Pieper FR. High-level expression of bovine αS1-casein in milk of transgenic mice. Transgenic Res 1998; 7:5-14; PMID:9556911; http://dx.doi.org/10.1023/A:1008892702466
[12] Yang P, Wang J, Gong G, Sun X, Zhang R, Du Z, Liu Y, Li R, Ding F, Tang B, Dai Y, Li N. Cattle mammary bioreactor generated by a novel procedure of transgenic cloning for large-scale production of functional lactoferrin. PLoS One 2008; 3:e3453; PMID:18941633; http://dx.doi.org/10.1371/journal.pone.0003453

[13] Kaushik R, Singh KP, Kumari A, Rameshbabu K, Singh MK, Manik RS, Palta P, Singla SK, Chauhan MS. Construction of a recombinant human insulin expression vector for mammalian gland-specific expression in buffalo (Bubalus bubalis) mammary epithelial cell line. Mol Biol Rep 2014; 41:5891-902; PMID:24969480; http://dx.doi.org/10.1007/s11033-014-3464-3

[14] Yang B, Wang J, Tang B, Liu Y, Guo C, Yang P, Yu T, Li R, Zhao J, Zhang L, Dai Y, Li N. Characterization of bioactive recombinant human lysozyme expressed in milk of cloned transgenic cattle. PLoS ONE 2011; 6:e17593; PMID:21436886; http://dx.doi.org/10.1371/journal.pone.0017593

[15] Kumar M, Keller B, Makalou N, Sutton RE. Systematic determination of the packaging limit of lentiviral vectors. Human Gene Therapy 2001; 12:1893-905; PMID:11589831; http://dx.doi.org/10.1089/10495398.2001.10303430.1753153947

[16] Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pleiffer A. Generation of transgenic cattle by lentiviral gene transfer into oocytes. Biol Reprod 2004; 71:405-9; PMID:15044266; http://dx.doi.org/10.1095/biolreprod.104.028472

[17] Itoh H, Ishihara Y, Maruyama K, Nishizawa S, Inoue S, Kamiya T, Endo M, Tsuchiya K, Yasumura Y, Nakai K. Generation of transgenic rabbits by pronuclear microinjection of a lentiviral vector. Front Biosci 2001; 6:i251-6; PMID:11496281; http://dx.doi.org/10.2741/i251

[18] Monzani PS, Sangalli JR, De Bem THC, Bressan FF, Fanteinato-Neto P, Pimentel JRV, Birgel-Junior EH, Fontes AM, Covas DT, Meirelles FV. Breeding of transgenic cattle for human coagulation factor IX by a combination of lentiviral system and cloning. Genet Mol Res 2013; 12:3675-8; PMID:23479170; http://dx.doi.org/10.1007/s11626-998-0009-x

[19] Monzani PS, Guemra S, Adona PR, Ohashi OM, Meirelles FV, Wheeler MB. MAC-T cells as a tool to evaluate lentivector construction targeting recombinant protein expression in milk. Anim Biotechnol 2015; 26:136-42; PMID:25380466; http://dx.doi.org/10.1080/10495398.2014.941468

[20] Park F. Lentivirus vector: are they the future of animal transgenesis? Physiol Genomics 2007; 31:159-73; PMID:17684037; http://dx.doi.org/10.1152/physiolgenomics.00069.2007

[21] Turchiano G, Latella MC, Gogol-Döring A, Cathoglio C, Mavilio F, Izsák Z, Ivics Z, Recchia A. Genomic analysis of Sleeping Beauty Transposon integration in human somatic cells. PLoS ONE 2014; 9:e112712; PMID:25390293; http://dx.doi.org/10.1371/journal.pone.0112712

[22] Ivics Z, Li MA, Mátés L, Boeke JD, Nagy A, Bradley A, Izsák Z. Transposon-mediated genome manipulation in vertebrates. Nat Protoc 2009; 6:415-22

[23] Ivics Z, Garrels W, Mátés L, Yau TY, Bashir S, Zidek V, Landa V, Geurts A, Praveneck M, Rülicke T, Kues WA, Izsák Z. Germline transgenesis in pigs by cytoplasmic microinjection of sleeping beauty transposons. Nat Protoc 2014; 9:794-809; PMID:24625779; http://dx.doi.org/10.1038/nprot.2014.010

[24] Ivics Z, Hiripi L, Hoffmann OI, Mátés L, Yau TY, Bashir S, Zidek V, Landa V, Geurts A, Praveneck M, et al. Germline transgenesis in rabbits by pronuclear microinjection of Sleeping Beauty transposons. Nat Protoc 2014; 9:794-809; PMID:24625779; http://dx.doi.org/10.1038/nprot.2014.009

[25] Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. Genetic transformation mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci USA 1980; 77:7380-4; PMID:6261253; http://dx.doi.org/10.1073/pnas.77.12.7380

[26] Schmidhauser C, Bissel MJ, Myers CA, Casperson GF. Extracellular matrix and hormones transcriptionally regulate bovine beta-casein 5′ sequences in stably transfected mouse mammary cells. Proc Natl Acad Sci USA 1990; 87:9118-22; PMID:2251252; http://dx.doi.org/10.1073/pnas.87.23.9118

[27] Monzani PS, Bressan FF, Mesquita LG, Sangalli JR, Meirelles FV. 5′-casein gene expression by in vivo cultured bovine mammary epithelial cells derived from developing mammary glands. Genet Mol Res 2011; 10:604-14; PMID:21491370; http://dx.doi.org/10.4238/ vol10-2gmr1034

[28] Febri RE, Pursel VG, Ruxroad GE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmer RD, Brinster RL. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 1985; 315:680-3; PMID:3892305; http://dx.doi.org/10.1038/315680a0

[29] van Berkel PHC, Welling MM, Geerts M, van Veen HA, Ravensbergen B, Salaheddine M, Pauwels EKJ, Pieper F, Nuijens JH, Nibbering PH. Large scale production of recombinant human lactoferrin in the milk of transgenic cows. Nature 2002; 41:5891-902; PMID:24969480; http://dx.doi.org/10.1007/s11033-014-3464-3
L, et al. Efficient production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. Proc Natl Acad Sci USA 2002; 99:14230-5; PMID:12393815; http://dx.doi.org/10.1073/pnas.222550299

[34] Zhang Y, Xi Q, Ding J, Cai W, Meng F, Zhou J, Li H, Jiang Q, Shu G, Wang S, et al. Production of transgenic pigs mediated by pseudotyped lentivirus and sperm. PLoS One 2012; 7:e53535; PMID:22536374; http://dx.doi.org/10.1371/journal.pone.0053535

[35] Eghbalsaeid S, Ghadek L, Laible G, Hosseini SM, Forouzanfar M, Hajian M, Obach F, Nasr-Esfahani MH, Obach M, et al. Exposure to DNA is insufficient for in vitro transgenesis of live bovine sperm and embryos. Reproduction 2013; 145:97-108; PMID:23137934; http://dx.doi.org/10.1530/RECP-12-0340

[36] Kanatsu-Shinohara M, Kato M, Takehashi M, Morimoto H, Takashima S, Chuma S, Nakatsuji N, Hirabayashi M, Shinohara T. Production of transgenic rats via lentiviral transduction and xenogenic transplantation of spermatogonial stem cells. Biol Reprod 2008; 79:1121-8; PMID:18685123; http://dx.doi.org/10.1095/biolreprod.108.071159

[37] Hadjantonakis AK, Macmaster S, Nagy A. Embryonic stem cells and mice expressing different GFP variants for multiple non-invasive reporter usage within a single animal. BMC Biotechnology 2002; 2:11; PMID:12079497; http://dx.doi.org/10.1186/1472-6750-2-11

[38] Iwasaki S, Campbell KHS, Galli C, Akiyama K, Iwasaki S. Production of live calves derived from embryonic stem-like cells aggregated to tetraploid embryos. Biol Reprod 2000; 62:470-5; PMID:10642589; http://dx.doi.org/10.1095/biolreprod62.2.470

[39] Furusawa T, Ohkoshi K, Kimura K, Matsuyama S, Akagi S, Kaneda M, Ikeda M, Hosoe M, Kizaki K, Tokunaga T. Characteristics of bovine inner cell mass-derived cell lines and their fate in chimeric conceptuses. Biol Reprod 2013; 89:28, 1-12

[40] Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Blackwell C, Ponce de León FA, Robl JM. Transgenic bovine chimeric offspring produced from somatic cell-derived stem like cells. Nat Biotechnol 1998; 16:642-6; PMID:9661197; http://dx.doi.org/10.1038/nbt0798-642

[41] Piedrahita JA, Moore K, Oetama B, Lee C-K, Scales N, Ramsandar J, Bazer FW, Ott T. Generation of transgenic porcine chimeras using primordial germ cell-derived colonies. Biol Reprod 1998; 58:1321-9; PMID:9603271; http://dx.doi.org/10.1095/biolreprod58.5.1321

[42] Imamura M, Hikabe O, Lin ZY-C, Okano H. Generation of germ cells in vitro in the era of induced pluripotent stem cells. Mol Reprod Dev 2014; 81:2-19; PMID:23996404; http://dx.doi.org/10.1002/mrd.22259

[43] Monzani PS, Guemra S, Zanin R, Lima M, Adona PR, Ohashi OM. Working with lentivirus for generation of transgenic bovine embryos. Pharm Anal Acta 2014; 5:e169

[44] Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmot I, Colman A, Campbell KH. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 1997; 278:2130-2133; PMID:9405350; http://dx.doi.org/10.1126/science.278.5346.2130

[45] Cibelli JB, Campbell KH, Seidel GE, West MD, Lanza RP. The health profile of cloned animals. Nat Biotechnol 2002; 20:13-14; PMID:11753346; http://dx.doi.org/10.1038/mbio.2002.0102-13

[46] Li S, Li Y, Du W, Zhang L, Yu S, Dai Y, Zhao C, Li N. Aberrant gene expression in organs of bovine clones that die within two days after birth. Biol Reprod 2005; 72:258-65; PMID:15240423; http://dx.doi.org/10.1095/biolreprod.104.029462

[47] Panarace M, Agüero JI, Garrote M, Jauregui G, Sevogia A, Cané L, Gutiérrez J, Marfil M, Rigali F, Pugliese M, et al. How healthy are clones and their progeny: 5 years of field experience. Theriogenology 2007; 67:142-51; PMID:17067665; http://dx.doi.org/10.1016/j.theriogenology.2006.09.036

[48] Zakhartchenko V, Mueller S, Alberio R, Schernthaner W, Stojkovic M, Wenigerkind H, Wanke RD, Lassnig C, Brem G. Nuclear transfer in cattle with non-transfected and transfected fetal or cloned transgenic fetal and postnatal fibroblast. Mol Reprod Develop 2001; 60:362-9; http://dx.doi.org/10.1002/mrd.1098

[49] Niemann H, Lucas-Hahn A. Somatic cell nuclear transfer cloning: Pratical applications and current legislation. Reprod Dom Anim 2012; 47:5-10; http://dx.doi.org/10.1111/j.1439-0531.2012.02121.x

[50] Salamone D, Barañao L, Santos C, Bussmann L, Artuso J, Werning C, Prync A, Carbonetto C, Dabsys S, Munar C, et al. High level expression of bioactive recombinant human growth hormone in the milk of a cloned transgenic cow. J Biotechnol 2006; 124:469-72; PMID:16716426; http://dx.doi.org/10.1016/j.jbiotec.2006.01.005

[51] Echelard Y, Williams JL, Destrempes MM, Koster JA, Overton SA, Pollock DP, Rapiejko KT, Behboodi E, Masiello NC, Gavin WG, et al. Production of recombinant human albumin by a herd of cloned transgenic cattle. Transgenic Res 2009; 18:361-76; PMID:19031005; http://dx.doi.org/10.1007/s11248-008-9229-9

[52] Kumar A, Singh S. Direct evolution: Tailoring biocatalysis for industrial application. Crit Rev Biotechnol 2013; 33:365-78; PMID:22985113; http://dx.doi.org/10.1080/07388551.2012.716810

[53] Adrio JL, Demain AL. Microbial cells and enzymes – A century of progress. In Methods in Biotechnology. Microbial Enzymes and Biotransformations; Barredo JL, Ed; Humana Press: Totowa, NJ, USA, 2005; 17:1-27.

[54] Kang Y, Jimenez-Flores R, Richardson T. Casein genes and genetic engineering of caseins. In Genetic Engineering of Animals, Springer US 1986; 37:95-111.