Transgenesis and gene edition in small ruminants

Transgênese e edição gênica em pequenos ruminantes

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Abstract

This review summarizes the main achievements with the use of transgenesis and genome editing technologies in sheep and goats. Transgenesis, also referred to as recombinant DNA (rDNA) technology, made possible by the first time 30 years ago the addition of novel traits from a given species into a different one. On the other hand, more recently genome editing appears a much more precise method of making changes to the genome of a plant, animal, or other living organism, allowing for the addition, substitution, or deletion of specific nucleotides in an organism's genome. With transgenesis, the introduction of new DNA into an organism's genome was generally without control of the site of the genome in which the insertion of that rDNA construct would occur. With genome editing in contrast, researchers and developers of products can make specific changes in precise locations of the genome. This concept was absolutely improved with the novel CRISPR/Cas system, making genome edition cheaper, more efficient, easier and affordable for every Laboratory around the world. This revolution that originally emerged from molecular biology and passed to biomedicine, has recently been applied to livestock and agriculture. In addition, the application of this technology in sheep, goats, pigs and cattle, also has been possible by the advance of assisted reproductive technologies for embryo production, micromanipulation, cryopreservation and transfer. In general, multidisciplinary approaches including basic research and technical improvements, participation of private actors and adequate regulation should be merged to take advantage of this potent biotechnology in different countries.

Keywords: genome edition, livestock, endonucleases, embryo, micromanipulation.

Introduction

Since ever the human being has tried to modify and adapt the traits of other species for his own benefit and progress. For that, traditional breeding and selection applied in livestock and agriculture has been the standard practice. One important breakthrough achieved during the 80's was the ability to change the genetic code of microorganisms, plants and animals in order to overexpress or silence a gene of interest, aiming at increasing the overall production or pest resistance, among other examples (reviewed by Uzogara, 2000).

In sheep and goats, genetic engineering has been used to improve productive traits mainly focused on milk, meat or wool production, as well as the generation of disease resistant animals for breeding and propagation. In addition, both species have been proposed as models to study human diseases or the generation of recombinant proteins in milk (reviewed by Menchaca et al., 2016).

Several techniques have been described and applied to modify the genome of these species (Menchaca et al, 2016), being more or less difficult to implement in productive herds. The available techniques include pronuclear DNA microinjection, somatic cell nuclear transfer (SCNT), lentivirus microinjection, RNA interference, sperm mediated gene transfer (SMGT), and more recently gene edition by the use of endonucleases, increasing the possibility to generate mutant animals as never seen before. Nucleases, and particularly the CRISPR/Cas system, make genome editing in animals cheaper, easer, more effective and less time-consuming than before.

During the last 30 years, sheep and goats have strongly contributed to the development of genetic modification technologies. The first report of a genetically modified farm animal was in sheep (Hammer et al., 1985). Also, after Dolly's birth, sheep were the first transgenic animal reported produced by nuclear transfer (Schnieke et al., 1997). Transgenic goats have been used as efficient bioreactors for the production of recombinant proteins in milk, mainly due to its short gestation period, ease of milking and outstanding protein synthesis capacity. The first drug derived from GE animals (ATryn®) approved for human use by the European Medical Agency in 2006 and the US Food and Drug Administration in 2009, was produced by goats (Adiguzel et al., 2009). Recently, with the arrival of the nucleases, it is believed that the regulatory pathway of drugs and products from gene edited animals will be much shorter and easier than before.

In this review we summarize the most relevant technologies used nowadays for the generation of genetically modified sheep and goats, with special emphasis in gene editing nucleases.

DNA pronuclear microinjection

The introduction and stable integration of a DNA fragment into the mammalian zygote was first achieved in mice (Gordon et al., 1980; Gordon and Ruddle, 1981). After these reports, several small ruminants' models

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have been developed using this technology. Among the published models, there are goats producing spider silk (Baldassarre et al., 2003), human butyryl-cholinesterase (Baldassarre et al., 2008), lysozyme (Maga et al., 2003), human tissue plasminogen activator (Ebert et al., 1991), human granulocyte colony stimulating factor (Ko et al., 2000; Freitas et al., 2007), human clotting factor IX (Huang et al., 2001), human lactoferrin (Zhang et al., 2008) and human antithrombin (Edmunds et al., 1998). Despite all these efforts, up to date only one product, recombinant antithrombin III (ATryn®) is available for human use since 2006. In that sense, some reports of risk assessment trying to validate the safety of this technology have been published (Steinkraus et al., 2012; Clark et al., 2014), indicating there is no risk of pleiotropic effects of fetal transgenic cells crossing the placental barrier.

On the other hand, few genetically modified sheep models developed using pronuclear microinjection have been reported. The preferred target for sheep is to enhance meat or wool production (Murray et al., 1989; Rexroad et al., 1989; Bawden et al., 1995; Damak et al., 1996; Adams et al., 2002), or generate disease resistant models (Clements et al., 1994). In addition, some models of human diseases have been also developed in sheep (Jacobsen et al., 2010).

The standard procedure in DNA pronuclear microinjection consist in the introduction of a purified DNA fragment into a zygote, usually up to 50 kb although cargo capacity has been increased with the use of bacterial artificial chromosomes (BAC) and yeast artificial chromosomes (YAC). Some picoliters of DNA are introduced into the pronucleus with the aid of micromanipulators, and the surviving embryos (~50%) are left in culture until transfer to synchronized recipients or frozen.

Although this technology was the method of choice to generate gain of function transgenic models, it is extremely inefficient at the end of the process, with less than 10% of the newborn carrying the desired mutation with unpredictable expression (Niemann and Kues, 2007). Moreover, the random integration and frequent incidence of mosaicism makes this approach not suitable for many research or industrial projects.

Somatic cell nuclear transfer

Several genetically modified sheep and goats have been obtained using this method widely applied after the birth of Dolly (Wilmut et al., 1997). Although the overall efficiency is relatively low (less than 10% of transferred embryos will born) and some developmental abnormalities has been reported, SCNT has proven to be extremely useful and is still applied until now. The main application of this technology is in biomedicine, mainly in pigs for xenotransplantation or the production of human recombinant proteins in milk of goats (Tan et al., 2016). Few models aiming to enhance livestock production or resist diseases have been reported.

Briefly, for transgenesis mediated by SCNT the desired DNA is firstly introduced into the host cell (fetal or adult) in culture by electroporation or lipid transfection, and the mutated cells are then selected using antibiotics and can be fully characterized before being introduced into enucleated oocytes to generate "reconstructed" NT embryos. One of the advantages of this method is that all the offspring born will be genetically modified, and that the site of integration, number of copies and integrity of the transgene had been characterized before.

Although the resultant living animals will 100% modified and fully characterized, the technique has several drawbacks that must be taken into account before considering its implementation in the laboratory. Among others problems, the correct selection of donor cells, cell cycle synchronization and the activation of cell-oocyte couplets must be carefully considered for an efficient epigenetic reprogramming, avoiding fetal abnormalities and placental problems (Niemann, 2016).

Lentiviral vectors

The first reports of the use of lentiviral vectors (LV) demonstrates that these vectors could be easily used as excellent carriers to integrate DNA into the host genome (Lois et al., 2002; Pfeifer, 2004). The advantages of using this technique are the easy injection into the perivitelline space without the need to visualize the pronuclei, the low rate of injury in embryos, and the high efficiency to rapidly produce genetically modified large animals. In addition, low gene silencing (Pfeifer et al., 2002), and stable integration could be achieved, making this approach appealing for many laboratories working in small ruminants species. On the other hand, some limitations should be considered before introducing this technology in the bench. This disadvantages include a limited cargo size (up to 8 kb) of the vectors, a single copy randomly integrated in multiple sites of the genome, and the not so easy production of high titer virus (Remy et al., 2010).

In sheep, this approach has proven to be very efficient in our laboratory, obtaining more than 95% of GFP embryos after LV microinjection and 100% (9/9) of GFP lambs born with almost 90% showing full expression (Crispo et al., 2015b).

Briefly, high concentration (10^9 particles/ml) LV construction is microinjected into the perivitelline space of one or two-cell embryos and immediately cultured *in vitro* until blastocyst for embryo transfer or cryopreservation. The ability of LV to reach the genome makes possible to inject the construction in both stages with high efficiency and low risk of mosaicism. In average, 200-300 embryos can be microinjected in one session, with almost 100% survivability rate, being thus an efficient approach. The effect of micromanipulation

on embryo development, if it exists, is minimum. When we compared embryo development of *in vitro* produced zygotes submitted or not to perivitelline microinjection with lentiviral vectors, no difference in blastocyst rate was found (Crispo et al., 2015b). In addition, the same study showed that pregnancy rate was similar among microinjected vs. non microinjected control zygotes. Thus, regarding our experience, the micromanipulation required for perivitelline injection seems to be minimally invasive for the embryo.

Endonucleases

Nowadays, several laboratories around the world are changing the strategy for the obtainment of genetically modified animal models in most of the species. This is in part due to the revolution of restriction endonucleases (DNA cutting enzymes) as gene target tools. These enzymes or genome editors recognizes specific DNA sequences with the ability to cut and generate double strand breaks (DSBs) at specific loci within the genome (Urnov et al., 2010; Tan et al., 2012; Doudna and Charpentier, 2014). The generated DBS trigger the hosts DNA repair mechanisms by two competing pathways (Kanaar et al., 1998), non-homologous end joining (NHEJ) and homology directed repair (HDR). This novel tool allow the generation of knock-out (KO) and knock-in (KI) large animal models, something difficult to achieve by homologous recombination in somatic cells in these species.

There are three different site-specific endonucleases used nowadays, of which CRISPR/Cas in the most revolutionary one due to its simplicity. Zinc Finger Nucleases (ZFN) were the first available tool, but the necessary reagents are difficult and expensive to be produced, and its construction challenge is restricting its use by the scientific community. ZFN has been applied in mice and rats, but there are no reports of genetically edited sheep and goats born yet.

On the other hand, TALENs, another restriction endonuclease similar to ZFNs, have been successfully used to produce myostatin genome edited sheep (Proudfoot et al., 2014). To obtain the modified animals, TALEN mRNA was microinjected into IVF zygotes obtaining 24% of blastocyst development rate. Pregnancy rate was 88.9% (8/9) and mutation rate 11.1% (1/9). The born lamb showed a heterozygous edition at the myostatin locus. Knock-in goats as also been successfully produced using TALEN together with SCNT. In this case, a β-Lactoglobulin-free milk goat has been obtained for temptative use as mammary gland bioreactors for the large-scale production of human lactoferrin in milk (Cui et al., 2015).

The third and more powerful genome editor reported four years ago is known as Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR associated gene 9 (Cas9). The difference with the previous two is the use of RNA instead of proteins to confer target specificity (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). It has many advantages over the previous tools: any molecular biology lab can design the construct, reagents are cheap and easy to make, multiplex editing is feasible and reported efficiencies are really high. In addition, cytoplasm mRNA microinjection makes this approach feasible in species were the pronucleus is difficult to visualize, as sheep.

The CRISPR/Cas system has been rapidly tested in several species, including sheep and goats. In sheep, CRISPR/Cas9 mRNA to target myostatin locus was microinjected into IVF derived zyogtes (Crispo et al., 2015a). In this study, we compared cleavage and development rates of microinjected zygotes using CRISPR/Cas9 vs. buffer control solution, showing similar results and demonstrating the innocuity of the procedure. The mutation rate was detected in 50% of embryos, and from 10 KO lambs born from 22 total lambs, eight of them were biallelic. Homozygous animals showed a strong double muscle phenotype as expected, and were heavier than their control wildtype counterparts.

In goats, four genes were disrupted simultaneously in caprine cells, which were then used for SCNT resulting in live-born goats showing biallelic mutations (Ni et al., 2014). This shows the power and easy of this approach to generate multiplex gene targeting in large animals.

Final remarks

Genetically engineered animals have been produced around the world since 30 years ago. Nevertheless, we are witnessing a true revolution regarding the way these models can be generated since the apparition of the site-specific endonucleases, mainly the CRISPR/Cas9 system in large animal species. This novel technology allows to obtain more refined animal models, including knock-outs and knock-ins, more rapidly and efficiently as never achieved before. For research groups working in livestock species, this system presents many more opportunities than the previous technologies, allowing the production of animal models in biomedicine, agriculture or basic research. The easy and non-expensive production of molecular tools, easy and fast cytoplasmic microinjection and the development of associated reproductive technologies such as *in vitro* embryo production, cryopreservation and embryo transfer, makes CRISPR/Cas system available to everyone.

On the other hand, few challenges must be overcome while the technology is still on development. A more efficient system to promote HDR instead of NHEJ should be still described, with the aim to produce KI models of interest. In that sense the use of NHEJ inhibitor compounds and RNAi strategies to suppress components of the NHEJ molecular machinery have been tested.

Also, off-target sites have chances to appear in similar site of the genome, and that should be further explored, although recent mouse studies indicate this is not of much concern (Iyer et al., 2015). To overcome this, among other strategies, the use of nickase variant enzymes has been reported (Frock et al., 2015) that could also promote HDR.

Another issue to consider is the mosaicism in founder animals. Using gene edited cells in combination with SCNT could be an alternative that may overcome this problem.

In summary, the easy and non-expensive production of molecular tools, easy and fast cytoplasmic microinjection, and the improvement of associated reproductive technologies, makes CRISPR/Cas system available to everyone. There will be a huge application of this promising tool in large animal species in the following years.

References

Adams NR, Briegel JR, Ward KA. The impact of a transgene for ovine growth hormone on the performance of two breeds of sheep. J Anim Sci, v.80, p.2325-2333, 2002.

Adiguzel C, Iqbal O, Demir M, Fareed J. European community and US-FDA approval of recombinant human antithrombin produced in genetically altered goats. Clin Appl Thromb Hemost, v.15, p.645-651, 2009.

Baldassarre H, Wang B, Kafidi N, Gauthier M, Neveu N, Lapointe J, Sneek L, Leduc M, Duguay F, Zhou JF, Lazaris A, Karatzas CN. Production of transgenic goats by pronuclear microinjection of in vitro produced zygotes derived from oocytes recovered by laparoscopy. Theriogenology, v.59, p.831-839, 2003.

Baldassarre H, Hockley DK, Dore M, Brochu E, Hakier B, Zhao X and Bordignon V. Lactation performance of transgenic goats expressing recombinant human butyryl-cholinesterase in the milk. Transgenic Res, v.17, p.73-84, 2008.

Bawden CS, Sivaprasad AV, Verma PJ, Walker SK and Rogers GE. Expression of bacterial cysteine biosynthesis genes in transgenic mice and sheep: toward a new in vivo amino acid biosynthesis pathway and improved wool growth. Transgenic Res, v.4, p.87-104, 1995.

Clark M, Murray JD, Maga EA. Assessing unintended effects of a mammary-specific transgene at the whole animal level in host and non-target animals. Transgenic Res, v.23, p.245-256, 2014.

Clements JE, Wall RJ, Narayan O, Hauer D, Schoborg R, Sheffer D, Powell A, Carruth LM, Zink MC, Rexroad CE. Development of transgenic sheep that express the visna virus envelope gene. Virology, v.200, p.370-380, 1994.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex Genome Engineering Using CRISPR/Cas Systems. Science, v.339, p.819-823, 2013.

Crispo M, Mulet AP, Tesson L, Barrera N, Cuadro F, Dos Santos-Neto PC, Nguyen TH, Creneguy A, Brusselle L, Anegon I, Menchaca A. Efficient Generation of Myostatin Knock-Out Sheep Using CRISPR/Cas9 Technology and Microinjection into Zygotes. PLoS One, v.10, p.e0136690, 2015a.

Crispo M, Vilarino M, Dos Santos-Neto PC, Nunez-Olivera R, Cuadro F, Barrera N, Mulet AP, Nguyen TH, Anegon I, Menchaca A. Embryo development, fetal growth and postnatal phenotype of eGFP lambs generated by lentiviral transgenesis. Transgenic Res, v.24, p.31-41, 2015b.

Cui C, Song Y, Liu J, Ge H, Li Q, Huang H, Hu L, Zhu H, Jin Y, Zhang Y. Gene targeting by TALEN-induced homologous recombination in goats directs production of beta-lactoglobulin-free, high-human lactoferrin milk. Sci Rep, v.5, p.10482, 2015.

Damak S, Su H, Jay NP, Bullock DW. Improved wool production in transgenic sheep expressing insulin-like growth factor 1. Biotechnology, (N Y), v.14, p.185-188, 1996.

Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science, v.346, p.1258096, 2014.

Ebert KM, Selgrath JP, DiTullio P, Denman J, Smith TE, Memon MA, Schindler JE, Monastersky GM, Vitale JA, Gordon K. Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. Biotechnology *(N Y)*, v.9, p.835-838, 1991.

Edmunds T, Van Patten SM, Pollock J, Hanson E, Bernasconi R, Higgins E, Manavalan P, Ziomek C, Meade H, McPherson JM, Cole ES. Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin. Blood, v.91, p.4561-4571, 1998

Freitas VJF, Serova IA, Andreeva LE, Dvoryanchikov GA, Lopes-Jr ES, Teixeira DIA, Dias LPB, Avelar SRG, Moura RR, Melo LM, Pereira AF, Cajazeiras JB, Andrade MLL, Almeida KC, Sousa FC, Carvalho ACC, Serov OL. Production of transgenic goat (Capra hircus) with human Granulocyte Colony Stimulating Factor (hG-CSF) gene in Brazil. An Acad Bras Cienc, v.79, p.585-592, 2007.

Frock R, Hu J, Meyers R, Ho Y, Kii E, Alt F. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. Nat Biotechnol, v.33, p.179-186, 2015.

Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci U S A, v.77, p.7380-7384, 1980.

Gordon JW, Ruddle FH. Integration and stable germ line transmission of genes injected into mouse pronuclei. Science, v.214, p.1244-1246, 1981.

Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL. Production of transgenic rabbits, sheep and pigs by microinjection. Nature, v.315, p.680-683, 1985.

Huang SZ, Huang Y, Chen MJ, Zeng FY, Ren ZR, Zeng YT. Selection of in vitro produced, transgenic embryos by nested PCR for efficient production of transgenic goats. Theriogenology, v.56, p.545-556, 2001.

Iyer V, Shen B, Zhang W, Hodgkins A, Keane T, Huang X, Skarnes W. Off-target mutations are rare in Cas9-modified mice. Nat Methods, v.12, p.479, 2015.

Jacobsen JC, Bawden CS, Rudiger SR, McLaughlan CJ, Reid SJ, Waldvogel HJ, MacDonald ME, Gusella JF, Walker SK, Kelly JM, Webb GC, Faull RL, Rees MI, Snell RG. An ovine transgenic Huntington's disease model. Hum Mol Genet, v.19, p.1873-1882, 2010.

Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. Elife, v.2, p.e00471, 2013.

Kanaar R, Hoeijmakers JH, van Gent DC. Molecular mechanisms of DNA double strand break repair. Trends Cell Biol, v.8, p.483-489, 1998.

Ko JH, Lee CS, Kim KH, Pang MG, Koo JS, Fang N, Koo DB, Oh KB, Youn WS, Zheng GD, Park JS, Kim SJ, Han YM, Choi IY, Lim J, Shin ST, Jin SW, Lee KK, Yoo OJ. Production of biologically active human granulocyte colony stimulating factor in the milk of transgenic goat. Transgenic Res, v.9, p.215-222, 2000.

Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science, v.295, p.868-872, 2002.

Maga EA, Sargent RG, Zeng H, Pati S, Zarling DA, Oppenheim SM, Collette NM, Moyer AL, Conrad-Brink JS, Rowe JD, BonDurant RH, Anderson GB, Murray JD. Increased efficiency of transgenic livestock production. Transgenic Res, v.12, p.485-496, 2003.

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE and Church GM. RNA-Guided Human Genome Engineering via Cas9. Science (New York, N.Y.), v.339, p.823-826, 2013.

Menchaca A, Anegon I, Whitelaw CB, Baldassarre H, Crispo M. New insights and current tools for genetically engineered (GE) sheep and goats. Theriogenology, 2016.

Murray JD, Nancarrow CD, Marshall JT, Hazelton IG, Ward KA. Production of transgenic merino sheep by microinjection of ovine metallothionein-ovine growth hormone fusion genes. Reprod Fertil Dev, v.1, p.147-155, 1989.

Ni W, Qiao J, Hu S, Zhao X, Regouski M, Yang M, Polejaeva IA, Chen C. Efficient gene knockout in goats using CRISPR/Cas9 system. PLoS One, v.9, p.e106718, 2014.

Niemann H, Kues WA. Transgenic farm animals: an update. Reprod Fertil Dev, v.19, p.762-770, 2007.

Niemann H. Epigenetic reprogramming in mammalian species after SCNT-based cloning. Theriogenology, v.86, p.80-90, 2016.

Pfeifer A, Ikawa M, Dayn Y, Verma IM. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci U S A, v.99, p.2140-2145, 2002.

Pfeifer A. Lentiviral transgenesis. Transgenic Res, v.13, p.513-522, 2004.

Proudfoot C, Carlson D, Huddart R, Long C, Pryor J, King T, Lillico S, Mileham A, McLaren D, Whitelaw CB, Fahrenkrug S. Genome edited sheep and cattle. Transgenic Research, p.1-7, 2014.

Remy S, Nguyen TH, Menoret S, Tesson L, Usal C and Anegon I. 2010. The use of lentiviral vectors to obtain transgenic rats. In: I. Anegon, editor, Rat Genomics. Humana Press, NY. p.109-125.

Rexroad CE, Jr, Hammer RE, Bolt DJ, Mayo KE, Frohman LA, Palmiter RD, Brinster RL. Production of transgenic sheep with growth-regulating genes. Mol Reprod Dev, v.1, p.164-169, 1989.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science, v.278, p.2130-2133, 1997.

Steinkraus HB, Rothfuss H, Jones JA, Dissen E, Shefferly E, Lewis RV. The absence of detectable fetal microchimerism in nontransgenic goats (Capra aegagrus hircus) bearing transgenic offspring. J Anim Sci, v.90, p.481-488, 2012.

Tan W, Carlson D, Walton M, Fahrenkrug S, Hackett P. Precision editing of large animal genomes. Adv Genet, v.80, p.37-97, 2012.

Tan W, Proudfoot C, Lillico SG, Whitelaw CB. Gene targeting, genome editing: from Dolly to editors. Transgenic Res, v.25, p.273-287, 2016.

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. Nat Rev Genet, v.11, p.636-646, 2010.

Uzogara S. The impact of genetic modification of human foods in the 21st century: A review. Biotechnology Advances, v.18, p.179-206, 2000.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature, v.385, p.810-813, 1997.

Zhang J, Li L, Cai Y, Xu X, Chen J, Wu Y, Yu H, Yu G, Liu S, Zhang A, Chen J, Cheng G. Expression of active recombinant human lactoferrin in the milk of transgenic goats. Protein Expr Purif, v.57, p.127-135, 2008.