

State of the art in the production of transgenic goats

H. Baldassarre^{A,C}, B. Wang^A, C. L. Keefer^B, A. Lazaris^A and C. N. Karatzas^A

^ANexia Biotechnologies Inc., 1000 Ave. St. Charles Block 'B', Dorion-Vaudreuil, Quebec J7V 8P5, Canada.

^BPresent address: Department of Animal & Avian Science, University of Maryland, College Park, MD 20742-2311, USA. °To whom correspondence should

be addressed, email: hbaldassarre@nexiabiotech.com

Abstract. This review summarises recent advances in the field of transgenic goats for the purpose of producing recombinant proteins in their milk. Production of transgenic goats via pronuclear microinjection of DNA expression vectors has been the traditional method, but this results in low efficiencies. Somatic cell nuclear transfer has dramatically improved efficiencies in rates of transgenesis. Characterisation of transfected cells *in vitro* before use in nuclear transfer guarantees that kids born are transgenic and of predetermined gender. Using these platform technologies, several recombinant proteins of commercial interest have been produced, although none of them has yet gained marketing approval. Before these technologies are implemented in goat improvement programmes, efficiencies must be improved, costs reduced, and regulatory approval obtained for the marketing of food products derived from such animals.

Extra keywords: nuclear transfer, pronuclear microinjection, recombinant proteins.

Introduction

The production of transgenic farm animals that contain exogenous DNA stably incorporated into their genome so that the 'transgene' is transmitted to the offspring in a Mendelian fashion has several applications. Besides the obvious scientific interest for the study of genes and their regulation, transgenic animal technologies have been proposed as a method to accelerate livestock improvement, by means of introducing new genes or modifying the expression of endogenous genes that regulate traits of economic importance (Wall *et al.* 1992; Wall 1996; Wheeler *et al.* 2003). However, to date, transgenic goats have only been generated for the purpose of producing valuable recombinant proteins of pharmaceutical interest.

Following the birth of the first cloned sheep (Wilmot *et al.* 1997), somatic cell nuclear transfer (SCNT) has been proposed as a method to clone high genetic merit animals and endangered species. The main utilisation of this technology, however, in goat species has been the generation of transgenic founders using λ -vzrr0-transfected cell lines with a DNA expression vector of interest, and the cloning of such transgenic animal founders (Baguisi *et al.* 1999; Keefer *et al.* 2001, 2002; Baldassarre *et al.* 2002, 20036).

The present manuscript reviews the methods used for production of transgenic and cloned goats, as well as the current and potential applications of these technologies.

Methods for transgenic goat production

Pronuclear microinjection and SCNT have been the two methods of choice for producing transgenic goats. Other

methods, such as sperm-mediated gene transfer (reviewed by Celebi *et al.* 2003), the use of recombinant viral vectors (Haskell and Bowen 1995; Hofmann *et al.* 2003), the establishment and use of embryonic stem (ES) cells and microprojectile cell bombardment technique, to name a few, may be possible in the future, but their application for generating transgenic goats has not been reported.

Pronuclear microinjection

Following the first report of generating transgenic mice produced by pronuclear microinjection almost 25 years ago (Gordon *et al.* 1980), transgenic rabbits, sheep, pigs (Hammer *et al.* 1985), cattle (Krimpenfort *et al.* 1991) and goats (Ebert *et al.* 1991) have been reported thereafter using the same technology. Until recently, pronuclear microinjection has been the method of choice for gene transfer in the production of transgenic goats. Although the method is reliable, efficiencies are low as a result of low integration rates (<10% of the kids born are transgenic). Furthermore, the random integration of transgenes in sites of the genome not transcriptionally active results in variable, unpredictable levels of expression (Wall *et al.* 1992; Wall 1996; Wheeler *et al.* 2003). Factors affecting the overall efficiencies of gene transfer using pronuclear microinjection are summarised in Table 1.

To date, transgenic goats have only been produced for the purpose of harvesting recombinant proteins of interest. For this application, protein production is targeted in the mammary gland owing to its outstanding protein synthetic capacity and the ease for milk collection. As a result, the

Table 1. Factors influencing the overall efficiencies of useful transgenic founders using the pronuclear microinjection method

- Response to superovulation treatment in donors
- DNA expression vector (purity, concentration)
- Pronuclei visibility in zygotes
- Timing of microinjection (early or late S-phase)
- Number of embryos cleaved and transferred following microinjection
- Species-specific patterns (i.e. high rates in mice, low in large species)
- Random integration of the transgene and failure of the founders to transmit or low transmission rates (mosaicism in germ cells)
- Failure of the founders to express at useful levels ($>1 \text{ gL}^{-1}$ of milk)
- Effect of the recombinant protein in the physiology of the mammary gland or animal

DNA sequence encoding the protein of interest is linked to a milk promoter, i.e. the regulatory sequence of a naturally occurring milk protein, such that the recombinant protein is expressed in the mammary gland during lactation. Both, the P-casein (Ebert *et al.* 1991) and the whey acidic protein promoter (Karatzas *et al.* 1999) have been reported for transgenic goat-milk-specific production of recombinant proteins.

In vivo produced zygotes have been traditionally used for the production of transgenic sheep and goats by DNA microinjection (Hammer *et al.* 1985; Clarke *et al.* 1989; Ebert *et al.* 1991; Baldassarre *et al.* 1999a; Echelard *et al.* 2000). For such purposes, donor goats are oestrus synchronised and superovulated, mated and/or artificially inseminated following oestrus detection, and subjected to surgical oviduct flushing for zygote collection. The procedure is characterised by a variable response in terms of the number of 'usable zygotes' for microinjection, as a result of variable ovulation rate, fertilisation rate and timing of ovulation/fertilisation, resulting in variable stages of development of the recovered embryos. The surgical nature of the technique limits the number of procedures performed on the same donor before surgical adhesions render the animal unusable for further embryo collections. Additionally, animal welfare guidelines may limit the number of laparotomies that can be practiced on the same animal.

The proportion of zygotes in the pronuclear stage at the time of collection can be improved by means of synchronizing ovulation with a GnRH injection administered 36 h after the removal of the medroxyprogesterone acetate-impregnated sponges used for oestrus synchronisation (Baldassarre *et al.* 1999b).

A further refinement of the pronuclear microinjection technology has been reported recently in using *in vitro* produced zygotes from oocytes recovered by laparoscopic ovum pick-up (LOPU; Baldassarre *et al.* 2002, 2003a). The improvements introduced by this method include a reliable response from donors in terms of oocytes recovered, the ability to repeat the procedure in the same animals at least

Table 2. Transgenesis rates via pronuclear microinjection using *in-vivo*- or *in-vitro*-derived zygotes

Zygote source	<i>In vivo</i>	<i>In vitro</i>
Zygotes microinjected & transferred	706	2001
Recipients transferred	186	311
Recipients pregnant	100 (54%)	157 (50%)
Kids born	115	211
Transgenic kids (% of kids born)	5 (4%)	15 (7%)
Transgenic kids (% of zygotes)	0.7%	0.7%

twice as many times as the laparoscopic procedure is significantly less traumatic, and better control over the stage of development of the zygotes at the time of microinjection (the technique can be planned to occur at a specific number of hours following IVF). The procedures for LOPU, IVM, IVF and microinjection have been described in detail elsewhere (Baldassarre *et al.* 1994, 2002; Wang *et al.* 2002). Briefly, donor goats are synchronised by means of intravaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix®, Upjohn Canada, Orangeville, Canada) inserted 10 days before LOPU, and gonadotrophin stimulated with 80mgNIH-FSH-Pl of Folltropin®-V (Bioniche, Belleville, Canada) and 300IU of equine chorionic gonadotrophin (eCG; Novormon®, Bioniche) in a single application 36h before LOPU. On the day of oocyte collection, donor goats are restrained on a standard laparoscopy table under general anaesthesia and follicles are aspirated under laparoscopic observation using a 20-G needle mounted in a plastic pipette connected to a collection tube and a vacuum line. *In vitro* maturation is carried out in 50-uL drops of tissue culture medium (TCM)-199 supplemented with hormones and 10% heat-inactivated estrous goat serum, at 39°C in a humidified incubator with 5% CO₂ in air for 24-27 h. *In vitro* fertilisation is performed in 50-uL drops of TALP medium supplemented with 20% heat-inactivated estrous goat serum and pre-capacitated fresh semen at a final concentration of 10⁶ sperm per mL. Following co-culture of the gametes for 15-20h, the presumptive zygotes are microcentrifuged to improve pronuclear visualisation and those with a visible pronucleus are microinjected with the designated DNA construct. Successfully microinjected zygotes are then transferred into the oviduct of recipient goats by mid-ventral laparotomy using a Tomcat® catheter (Sovereign-Sherwood, St Louis, MO, USA). Results from our group's pronuclear microinjection programmes using *in vivo* and *in vitro*-derived zygotes are shown in Table 2.

Contrary to data in bovine species (Krimpenfort *et al.* 1991), transgenesis rate and the rate of zygotes microinjected resulting in transgenic kids were similar regardless of the source of zygotes (*in vivo* or *in vitro*). Additionally, by increasing the number of zygotes transferred per recipient (average 3.8 v. 6.4), in order to compensate for the expected lower developmental capacity of the *in vitro* produced zygotes,

pregnancy rates achieved were similar. The data from Table 2 indicates that from 326 kids born, only 20 were transgenic (6%), which is indicative of the overall inefficiency of the system.

Somatic cell nuclear transfer

Following the birth of the first cloned sheep (Wilmut *et al.* 1997), SCNT using *in vitro* transfected cells with DNA expression vectors has been implemented in the production of transgenic sheep (Schnieke *et al.* 1997), cattle (Cibelli *et al.* 1998) and goats (Keefer *et al.* 2001).

Using this method, the DNA is randomly incorporated into the genome using selective pressure. However, the transgenic cells can be fully characterised (site of integration, number of integrated copies and integrity of the transgene) before use in nuclear transfer (NT). As a result, although the developmental capacity of 'reconstructed' NT-embryos is lower, the majority of the kids born are transgenic. This results in making this technology much more efficient than pronuclear microinjection.

Procedures for the production of transgenic founder goats from z'-vzzro-transfected cell lines have been described in detail elsewhere (Keefer *et al.* 2001, 2002; Baldassarre *et al.* 2003b). Briefly, fetal fibroblasts (FF) isolated from Day 27 to 30 fetuses are used for transfection of DNA expression vectors using lipid-mediated gene transfer. Single colonies are selected following culture in G418- (Geneticin®, Invitro-gen, Carlsbad, CA, USA) supplemented medium for 8-12 days. There is considerable controversy as to whether it is better to synchronise donor cells in a growtharrested state (GO) or in a proliferating state (G1). Serum starvation (low serum conditions) used to synchronise cells in GO can have detrimental consequences, such as induction of DNA fragmentation (Kues *et al.* 2000); however, other authors have found no differences in SCNT efficiencies when using serum starved v. growing cells (Peumetal. 2003). Alternatively, cells can also be synchronised by prolonged culture at confluency (GO arrest). Matured (Mil staged) oocytes are stained with Hoechst 33342, enucleated under short exposure to ultraviolet light (UV) light, and used as recipient cytoplasts. Individual donor cells are transferred into the perivitelline space of the enucleated oocytes, followed by fusion of the oocyte-cell couplets using an electric pulse (15 μ sec, 2.44 kVcnU¹) that can be repeated, if needed, 3 0-60 min later. Two to three hours after fusion the reconstructed embryos are activated using calcium ionomycin and cycloheximide. Finally, embryos are cultured overnight in G1.2 medium (Colorado Center for Reproduction Medicine, Englewood, CO, USA) and transferred on the following morning into the oviduct of recipient goats synchronised in Day 1 of their cycle. Our standard procedure is to transfer the embryos to recipients within 24 h from fusion, in order to avoid poor/abnormal development associated with *in vitro* culture of embryos (McEvoy *et al.* 2000). Alternatively, the nuclear transfer embryos can be

further cultured *in vitro* until they reach uterine stages of development. Several media have been successfully used for the *in vitro* culture (IVC) of goat embryos to the blastocyst stage, including synthetic oviduct fluid (SOF; Keskinetepe *et al.* 1998; Cognie *et al.* 2003) and Gardner's sequential media G1-G2 (Koeman *et al.* 2003).

Traditionally, *in vivo* matured oocytes collected from superovulated donors by surgical oviduct flushing are used as recipient cytoplasts (Baguisi *et al.* 1999). The use of *in vitro* matured oocytes obtained by LOPU from donors primed with gonadotrophins has been proposed as an improvement to the overall NT efficiencies in goats (Keefer *et al.* 2001, 2002; Baldassarre *et al.* 2002, 2003b). The system allows the use of donor goats at least twice as many times owing to the less traumatic nature of the procedure when compared to laparotomy-based oviduct flushing to collect ovulated oocytes. Additionally, it avoids problems resulting from variability with stage of development of *in vivo* matured oocyte recovery. A recent demonstration (Peura *et al.* 2003) that there were no differences in the use of *in vitro* v. *in vivo* matured oocytes in sheep nuclear-transfer programmes may indicate that there is little, if any, advantage in using *in vivo* matured oocytes as recipient cytoplasts.

Somatic cell nuclear transfer can also be implemented in the cloning of valuable animals such as transgenic founder goats. For such purpose, cells are typically obtained from the donor animal by skin biopsy (skin fibroblasts), or cumulus-granulosa cells recovered during oocyte collection (LOPU) of prepubertal transgenic goats (Baldassarre *et al.* 2002). Results obtained by our group using *in vitro* transfected FF for transgenic goat founder generation and adult cells for propagating such transgenic founders using skin fibroblasts and cumulus-granulosa cells, are shown in Table 3.

Another source of variation in the success of pregnancy establishment and maintenance, as well as neonatal viability, appears to be the cell line used in NT. Rates for different cell lines used (Table 3) ranged from 0 to 89% for initial pregnancy; 0 to 67% for pregnancy after 60 days; 0 to 67% for maintenance of pregnancy to term; and 0 to 67% perinatal kid mortality. Factors affecting the development of NT-reconstructed embryos include, among others, insufficient nuclear reprogramming. Following fusion of donor cells with the enucleated eggs, the nuclei are required to regain full ability to direct cell differentiation, otherwise some genes may not be correctly regulated in the resulting NT animals. In agreement with this, Jones *et al.* (2003) found a set of 39 genes to be differentially expressed in the livers of NT fetuses compared to standard embryo transfer (ET)-derived bovine fetuses.

Although most term pregnancies resulted in healthy kids, diverse types of abnormalities affecting the heart, liver and/or kidneys were observed following necropsy of those clones that were born dead or died shortly after birth. Live kids from some lines have manifested behavioural abnormalities

Table 3. Cumulative results of nuclear transfer in goats using laparoscopic ovum pick-up-sourced *in-vitro-matured* oocytes and either *n-v/fr-o*-transfected fetal fibroblasts or cumulus-granulosa cells and adult fibroblasts obtained from transgenic goats

	Transfected - FF		From TG animal		Total	
No. cell lines	37		20		57	
Oocytes processed	7516	(12.4/donor)	6377	(13.7/donor)	13893	(12.8/donor)
Embryos transferred	3528		3082		6610	
Recipients transferred	313	(11.2/ recip.)	279	(10.7/ recip.)	592	(11.1/ recip.)
Initial pregnancy	110	(34%)	102	(34%)	212	(36%)
Early pregnancy loss	36	(33%)	21	(20%)	57	(27%)
Late pregnancy loss	7	(6%)	4	(4%)	11	(5%)
Pregnancy to term	67	(21%)	77	(24%)	144	(24%)
Total kids born	111	(1.7/ recip.)	108	(1.4/ recip.)	219	(1.5/ recip.)
Total live kids	96	(86%)	78	(72%)	174	(80%)
Total live TG kids	84	(87%)	66	(85%)	150	(86%)

FF, Fetal fibroblasts; LOPU, laparoscopic ovum pick-up; TG, transgenic.

Table 4. Recombinant proteins produced in transgenic goats

Protein	Indication	Company	Stage of development
hAT III	Heparin-resistance	GTC	Phase III
HtPA	Acute heart infarction, thrombosis	Nexia	Pre-clinical
Monoclonal antibodies	Several including cancer and AIDS	GTC	Pre-clinical
a-Fetoprotein	Autoimmune diseases	GTC	Phase I
Spider silk	Sutures, prosthesis, etc.	Nexia	Pre-clinical
Malaria antigens	Malaria prevention (vaccination)	GTC	Pre-clinical
Butyryl-cholinesterase	OP intoxication	Nexia	Pre-clinical

AIDS, Acquired immune deficiency syndrome; hAT III, human antithrombin III; htPA, human tissue plasminogen activator; OP, organo-phosphates. GTC Biotherapeutics. Source: www.nexiabiotech.com (accessed April 2004) and www.gtc-bio.com (accessed April 2004).

(e.g. 'aggressive feeding'), while clones from some other lines have shown susceptibility to contracting respiratory infections. In most cases, these conditions improved dramatically and/or disappeared after 1 year of age (Baldassarre *et al.* 2003£).

Based on the results described above, in order to produce the 84 NT transgenic kids (generated by SCNT using transfected cell lines) one would have required to produce >1000 kids by pronuclear microinjection. Such calculation clearly illustrates the advantages introduced by the use of SCNT in transgenic founder generation programmes.

Application of transgenic and cloning technologies

Several applications of transgenic animal technology to livestock improvement have been proposed and reported in other species. Examples of such applications include: improved wool production in sheep expressing a keratin-IGF construct in wool follicles (Damak *et al.* 1996); increased growth/feed conversion in pigs carrying a metallothionein-growth hormone transgene (Nottle *et al.* 1999); lysostaphin expression in the mammary gland of dairy animals to confer protection against staphylococcal mastitis (Kerr *et al.* 2001); expression of a bacterial phytase gene in the salivary

glands of pigs for improved digestion of phytates resulting in better phosphorus utilisation and a significant reduction of phosphorus pollution in manure (Golovan *et al.* 2001); altering milk composition to increase piglet growth and health (Wheeler *et al.* 2001).

However, the use of transgenic technology in goats has only been reported in 'pharming' applications, i.e. the use of transgenic goats as bioreactors for the production of valuable recombinant proteins of pharmaceutical and biomedical interest. In that sense, goats offer a significant advantage over sheep as they are efficient milk producers (500 to 1000L of milk per lactation) and compared to cattle they offer lower maintenance cost, earlier sexual maturity and shorter pregnancy duration. Wall *et al.* (1992) estimated that the cost of producing a transgenic cow by pronuclear microinjection was US\$546 000 compared to US\$60 000 to produce a transgenic sheep or goat. Hence, goats are a suitable animal for the production of recombinant proteins required in quantities of several hundreds of kilograms per year, whereas transgenic cows are more suitable for proteins required in larger quantities, such as human serum albumin with an estimated demand of several metric tonnes per year. Recombinant proteins that have been expressed in the milk of transgenic goats for commercial purposes are summarised in Table 4.

Of all these products, the most advanced in the regulatory process for approval before marketing is recombinant human antithrombin III (rhATIII) (www.gtc-bio.com).

The use of the SCNT technology in goats has only been applied in the generation and propagation of transgenic animals expressing recombinant proteins of pharmaceutical interest. However, the use of SCNT for cloning of elite animals of proven production is obvious and may become a standard procedure in the future, once efficiencies are improved, costs reduced and regulatory clearance for human consumption is obtained.

Conclusions

The efficiencies in the production of transgenic goats have been dramatically improved in the last few years, especially as a result of the use of SCNT. The efficiencies have been sufficient to allow a few groups to produce recombinant proteins of pharmaceutical and biomedical interest, for commercial applications. When companies leading the application of this technology succeed in bringing products to the market, there is the potential for a significant increase in the demand of dairy goats for 'pharming' purposes.

Genomics, proteomics and bioinformatics offer the potential to identify genes or loci regulating traits of economic importance, which could be used as gene transfer candidates by transgenic technologies. Production traits that are proposed as eligible for transgenic modification include increased growth rate and improved carcass composition, improved feed utilisation, modified milk composition (higher in caseins for better cheese yield, lower in lactose, etc.), improved mohair production (higher fibre production and/or better textile properties such as softness, strength, etc.), improved reproductive performance (e.g. higher prolificacy) and increased disease resistance. In addition to a better understanding of the genes regulating these traits, a rather dramatic improvement in the efficiency of production of transgenic and cloned goats is necessary before these technologies can be implemented for livestock improvement programmes. The availability of ES-like cells for large farm animals in combination with SCNT will allow specific modification, deletion or addition of genes and loci, whereas pronuclear microinjection is currently limited to the addition of genes. Equally important, transgenic and cloned animals are under intense investigation by regulatory authorities to establish the safety of allowing derived products (meat, milk, etc.) for human consumption, a key step before any of these technologies is implemented at the farm level.

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