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Recent advances in micromanipulation and transgenesis in domestic mammals.

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ABSTRACT

***Background*:** Intracytoplasmic sperm injection (ICSI) involves mechanical transfer of a single sperm cell into ooplasm. A new application has been recently found for ICSI, the production of transgenic animals. Since the birth of ''Dolly'', the first adult somatic cloned mammal, viable offspring has been produced by nuclear transfer in many species including cattle. The present review briefly summarizes our experience with ICSI and somatic cell nuclear transfer mainly to produce transgenic embryos, as well as for the generation of new micromanipulation technique.

***Review*:** We have evaluated different factors that affect SCNT and transgenesis including the chemical activator, the transfection event and the effect of recloning. Also, we included a brief description of the ICSI technique, which we used in five different species, examining its potential to produce transgenic embryos. Finally different strategies to produce transgenic animals were analyzed: ICSI- mediated gen transfer (ICSI-MGT), Injection of cumulus cell and ooplasmic vesicle incubated for 5 min with the transgene or injection of the plasmid alone. All of them were very efficient in exogenous DNA expression at embryo stages but resulted in mosaic embryos. We demonstrated that "ICSI-MGT" assisted by chemical activation is the only treatment of sperm mediated gen transfer capable to generated transgenic embryos in ovine. Besides, after ICSI-MGT, it is possible to obtain enhanced green fluorescent protein (EGFP)-expressing embryos in five diferent species: ovine, porcine, feline, bovine and equine. Our studies also established for the first time that short term transgene co-incubation with somatic cells can produce transgene-expressing mammalian SCNT embryos, and also that parthenogenic, eDNA- expressing embryos can be obtained by injection of vesicles or eDNA alone. Moreover, eDNA--expressing embryos can be also obtained by cytoplasmic injection of vesicles in IVF zygotes, simplifying the traditional IVF pronuclear injection technique. We tried a further simplification of the technique in bovine oocytes and zygotes, by intracytoplasmically injecting them with eDNA-liposomes complexes. Approximately 70% of the cleaved embryos and 50% of the blastocysts expressed EGFP, when *egfp*–liposome was injected 16 h post-fertilization. Different approaches were assayed to reverse the mosaicism including a novel technique of gamete cloning. Our first approach consisted of the production of transgenic IVF embryos by vesicle microinjection to generate transgenic blastomeres to be used as donor cells for cloning. A high efficiency in mosaicism reversal and multiplication of transgenic embryos was attaineded. Other technique assayed was the separation of transgenic blastomeres followed by the aggregation of two-cell fused embryos or by the asynchronous younger blastomere successfully multiplied transgenic embryos, and theoretically reduces mosaicism rates in future offspring [15]. This technology can also be used to multiply embryos from animals with high genetic value. We demonstrated that a sperm and oocyte can be efficiently cloned. Green haploid androgenic blastomeres produced with the injection of a single sperm by *egfp* ICSI-MGT could be used to fertilized oocytes resulting in several homogeneous expressing embryos. 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 review summarizes recent experiments in micromanipulation and gene transfer in domestic animals. The objective is not to exhaustedly describe the research done in this field but to present the promising methods recently developed or evaluated in our lab.

***Conclusion*:** Significant advancements have been made in the course of the recent years in micromanipulation and transgenesis techniques. In our lab we have been evaluating ICSI and Nuclear transfer mainly to produce transgenic embryos. We used also transgensis to apply or developed new micromanipulation technique in domestic animals linke sperm and oocyte cloning.

Keywords: Micromanipulation, transgenesis, ICSI, cloning, nuclear transfer.

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I. INTRODUCTION

II. CLONING BY NUCLEAR TRANSFER

Cloning by somatic cell nuclear transfer has raised enormous interest; mainly it has made possible the propagation of elite domestic animals and the enginering of transgenic animals for agricultural and biomedical purposes. Briefly, nuclear transfer (NT) involves the enucleation of a recipient oocyte, followed by the transfer of a donor cell to the perivitelline space in close apposition of the recipient cytoplast, and their fusion. Development is induced artificially by chemical or physical activation. Production of cloned offspring by somatic cell nuclear transfer has been successfully attained in sheep [5,50,53], goats [1] and cows [8,17,50].

There are several factors that influence the results of NT including the methods of enucleation [26], fusion, activation and donor-recipient cell cycle synchrony. High efficiencies in enucleation of recipient oocytes have been achieved using DNA specific vital dyes to visualize chromatin [51,41]. Fusion of the donor cell with the recipient oocyte depends on the accuracy of cell alignment in the pulse field, contact of the donor cell with the recipient oocyte and size of the donor cells [9]. Nucleus can also be injected [6]. Activation of NT reconstructed embryos has been refined and rates of development into blastocysts are equivalent to *in vitro* fertilized oocytes [21].

Successful development of NT embryos has been accomplished using mature oocytes [52], zygote [24] and cleavage-stage embryos [44] as recipient cytoplast. However, this is dependent on the source of the donor nucleus. Compatibility of the cell cycle between the recipient cytoplasts and the donor cell is one of the important factors that influence development of NT embryos. Appropriate synchronization is necessary to preserve the ploidy of the reconstituted embryo.

A problem with existing nuclear transfer systems is that the survival of cloned embryos and fetuses is low. One possible explanation is that the donor nucleus may not be competent to support normal development. Somatic cells have been programmed to differentiate into a particular cell; its nucleus is programmed to transcribe a specific set of RNAs that are translated into a set of proteins that results in the cell having all the characteristics of its type. After nuclear transfer the nuclei condense, and many proteins that bind to chromatin and regulate transcription must be released. Other proteins may bind at the time of activation to induce decondensation. Reconstructed embryos can also present errors in genomic imprinting. The modification or disruption of genomic imprinting in early development may cause genetic diseases. Embryo imprinting was evaluated by several authors and may represent a problem for embryo or fetal survival during reconstruction [14,33].

A new compound Dehydroleucodine (DhL) was evaluated as a chemical activator to produce SCNT reconstructed embryos [46]. Results showed that DhL induces pronuclear formation dynamics more similar to IVF than 6-dimethylaminopurine (6-DMAP). Blastocyst development was higher after activation with this new compound combined with Citochalasyn B. Moreover, all DhL treatments induced polyploidy rates lower than Ionomycin followed by 6-DMAP, but cloned blastocyst rates statistically similar [6].

One of the main applications of SCNT is the cloning of animals of genetic value. We assessed a new alternative cloning technique, previously described in the bovine [34], 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. The sizes of vesicles and embryos *in vivo* were normal for all groups, and *in vitro* development of aggregated embryos beyond day 7 resulted in the highest embryo viability. Cloning equines by means of aggregation of two or three embryos does

not imply extra oocytes, and it is a good strategy to improve *in vitro* embryo development without alterations in *in vivo* progress. The first living cloned foal obtained by this method was born from two aggregated embryos On August 4, 2010 [11].

III. CLONING BY NUCLEAR TRANSFER USING A TRANSFECTED CELL LINE

After nuclear transfer was developed, microinjection tended to be replaced by nuclear transfer using genetically modified somatic donor cells [39,8]. A cow capable of expressing human growth hormone (hGH) was delivered in South America in September of 2002. The hGH production in milk from this animal was evaluated and results were published by Salamone *et al.* [38] in 2006. It was demonstrated that hGH can be produced at a large scale in the milk of transgenic cows. Only about 15 animals would be necessary to meet the worldwide requirements of this protein for the treatment of dwarfism in GH deficient children [38]. However, nuclear transfer as was described previously using genetically modified somatic donor cells presents a low overall efficiency, explained partly by epigenetic reprogramming failure [36].

Although, cloning is one of the most powerful techniques available to generate transgenic animals, several additional problems appear during clone production. For example, different transfection events of the same somatic cell line can affect embryo and/or fetal survival. In one experiment performed for a Biotechnology company, a fetal cell line was established from a 75-day-old Jersey female fetus, which was used as control and was also transfected 3 times with the same protocol. They were named Transfection 1, 2, and 3. Genetically modified cells were produced and isolated after selection with geneticin for 10–15 days following liposome transfection with a DNA construct containing a selectable neomycin resistance gene.

Results of embryo and fetal development are presented in table 1. One birth was obtained from the control. Four and 7 births were obtained from Transfections 1 and 3, respectively. Although Transfection 2 had good *in vitro* development, this treatment did not produce any pregnancies. This fact demonstrated that the transfection event provides an additional source of variability in obtaining live transgenic animals. Our results pointed out the necessity to monitor fetal survival by ultrasonography in order to detect any deficiencies in development introduced by transfection as soon as possible.

Table 1. Effect off different transfection events on same line in embryo and fetal survival.

Treatment	n	Blastocyst (%)	Implanted Recipients	Preg. 30d (%)	Birth
Control	197	122 (62)	33 (18)	4	1
Transfection 1	130	106 (82)	28 (22)	5	4
Transfection 2	137	96 (70)	34 (24)	0	0
Transfection 3	470	282 (60)	71 (15)	12	7

Percentages within columns with different superscripts are different ($P < 0.05$)

In a large scale cloning program destined to obtain transgenic animals, it is very important to produce well-characterized transgene integration and gene expression. However, after non-homologous transfections a wide variety of transgene copy numbers are introduced in different chromosome locations. Recloning a selected first-generation of transgenic calves offers the opportunity to increase the homogeneity among transgenic animals. Calf recloning was performed in an experiment (n= 1739 cloning procedure) in which the survival rate was evaluated after a second round of cloning from transgenic umbilical cord and ear calf fibroblast cell cultures. Seven births were obtained from the original fetal cell line, one birth was obtained from recloned umbilical cord cells and two calves from recloned ear fibroblasts. Development to blastocysts was different between transfected fetal fibroblasts and both recloned treatment groups. Differences were observed in pregnancy rates between blastocysts generated by the different sources of donor cells. Although it results in lower blastocyst production, our results suggest that recloning provides an additional method to obtain

transgenic animals, where fibroblasts from umbilical cord tissue could give better results for recloning than those obtained from young calf ear cells.

IV. INTRACYTOPLASMIC SPERM INJECTION (ICSI)

ICSI has been used in humans and mice [28,18]. In these species, sperm cell injection causes oocyte activation [27]. However, after ICSI most domestic animals do not develop appropriately [23,22]. Chemical activation protocols for ICSI frequently use ionomycin, a calcium ionophore, followed by 3 h of incubation previous to 6-DMAP treatment [35]. Recently, we produced a high rate of ICSI blastocysts in a domestic cat without chemical assisted activation, simplifying the procedure, and allowing for its application in the wild cat [25]. Most groups perform ICSI using a piezo-drill, but we recently obtained good blastocyst rates using chemical activation protocols, producing a live lamb Fig. 2 [31]. In equines this technique has great potential, because although oocytes can be recovered *in vivo* via ultrasound guided transvaginal aspiration, they are impossible to fertilize by regular *in vitro* protocols. ICSI appears to bypass this problem and we recently generated good blastocyst rates in the equine.

V. ICSI MEDIATED GENE TRANSFER

Several authors published techniques alternative to somatic cell nuclear transfer and pronuclear microinjection to obtain transgenic embryos and offspring including: Laparoscopic Insemination (LI) [19], *In Vitro* Fertilization (IVF) [20] and ICSI [32]. By LI and IVF, the possibility of generating transgenic animals is simple. However the use of these techniques has been in substantial debate [20,4]. On the other hand, ICSI is an efficient technique to produce offspring in the murine [32].

One of the limitations of ICSI-mediated gene transfer is that it results in a high frequency of mosaic expression of the transgene. A possible explanation for this is that the transgene is not integrated in the embryo genome before the first cell divisions [40,32,43,16]. Moreover, the transgene could remain extra-chromosomal and be lost during successive mitotic divisions, as was already shown in two previous reports [7].

In one experiment, we used "ICSI-MGT" assisted by chemical activation in five species, ovine, porcine, feline, bovine and equine, demonstrating that it is possible to obtain enhanced green fluorescent protein (EGFP)-expressing embryos in all of them. The spermatozoa of the five species were coincubated with pCX-EGFP plasmid and injected into the MII oocyte. The chemical activation protocol was ionomycin followed 3 hours later by 3 hours of treatment with 6-DMAP. We detected high proportions of the fluorescent EGFP embryos in all five species (23 to 60%) at day four and produced green blastocysts in bovine, ovine and cat [30].

In cattle, ICSI- mediated gene transfer was not evaluated until a report by Pereyra *et al.* [30] in 2008. The main reason for this is the poor outcome after conventional ICSI in this species. In another study we looked at the best conditions for intracytoplasmic ICSI-MGT in cattle. Various aspects of fertilization and embryonic development were assessed after five activation treatments. Spermatozoa were co-incubated with pCX-EGFP plasmid and injected into metaphase II oocytes, which were then treated with ionomycin (Io) before further activation with the following agents: 6-DMAP (Io-DMAP), additional Io plus 6-DMAP (2Io-DMAP), Io alone (2Io), ethanol (Io-EtOH), or strontium chloride (Io-SrCl2). Fertilization rates at 16 h after ICSI, presence of a condensed spermatozoon head on Day 4, and blastocyst and EGFP expression rates on Day 7 were evaluated. Fertilization rates did not differ significantly among treatments. All (100%) of EGFP-positive embryos resulted from ICSI fertilization, whereas at least 60% of EGFP-negative embryos had a condensed sperm head. Blastocyst rates after 2Io-DMAP were not significantly different from Io--DMAP or Io-EtOH, but they were higher than 2Io or Io-SrCl2 treatments (25.9, 18.7, 14.7, 9.4, and 10.9% respectively). In bovine, ICSI-MGT proved to be a powerful technique because over 80% of the blastocysts expressed EGFP protein [2]. We evaluated LI, IVF and ICSI to produce egfp-expressing ovine embryos, using spermatozoa previously exposed to pCX-EGFP plasmid. High cleavage and morulae/blastocysts rates were obtained with LI and IVF, but no egfp-expressing embryos resulted. In contrast, 91.6% egfp-expressing morulae and blastocysts were generated by ICSI [29].

VI. GENE TRANSFER BY NUCLEAR MICROINJECTION

The first method reported to produce transgenic animals was the microinjection of foreign DNA into the male pronuclei of zygotes [12]. Transgenesis by microinjection is still used mainly for research purposes in mice. This procedure has proven to be efficient in species such as rabbits, sheep and pigs

[13], but it is quite dependent on proper male pronuclei visualization and in some species, like the bovine, the efficiency is very low [10].

VII. GENE TRANSFER BY CYTOPLASMIC MICROINJECTION

Based in our results with ICSI, we tested if the sperm cell was a special cell to transfer the transgene, or if other cell types could also act as vectors. Surprisingly, when we injected cumulus cells or oolemma vesicles previously incubated with eDNA, and naked eDNA into the cytoplasm of MII oocytes we observed expression of eDNA [29]. Using confocal microscopy interaction, an interaction of FITC- labeled eDNA with cumulus cells and vesicles was demonstrated. In contrast, oocytes injected with DNA alone did not show signs of transgene accumulation, and their eDNA expression rates were lower. In a further experiment, we evaluated if transgene-expressing embryos could be produced by means of vesicle injection followed by IVF. Preliminary FISH analysis indicated detectable integration events in 1/5 of SCNT blastocysts treated. Our studies demonstrate for the first time that short term transgene co-incubation with somatic cells can produce transgene-expressing mammalian SCNT embryos, and also that parthenogenic, eDNA- expressing embryos can be obtained by injection of vesicles or eDNA alone. Moreover, eDNA-expressing embryos can be also obtained by cytoplasmic injection of vesicles in IVF zygotes, simplifying the traditional IVF pronuclear injection technique.

In other experiments different approaches were studied to improve transgenesis efficiency and to avoid mosaic expression patterns of transgene-expressing embryos. Circular and linear plasmid structures and cell cycle inhibitors (6-DMAP, and DhL) were tested to this aim [3]. *Egfp* expression was higher for linear than circular pCX-EGFP, and green blastocyst rates were higher for the groups inoculated with linear transgene incubated with vesicles than for free linear plasmid alone. FISH analysis showed integration evidence in green embryos. The cell cycle inhibitor 6-DMAP increased phosphorylated histone H2AX foci area, which mark DNA double stranded breaks and reduced mosaic expression.

We tried a further simplification of the techniqe in bovine oocytes and zygotes, by intracytoplasmically injecting them with eDNA-liposomes complexes. Approximately 70% of the cleaved embryos and 50% of the blastocysts expressed EGFP, when *egfp*–liposome was injected 16 h post-fertilization [48].

The percentage of integration of all these methods remains to be confirmed.

VIII. DIFFERENT APPROACHES FOR EMBRYO MULTIPLICATION AND MOSAICISM REVERSION

Our first approach consisted of the production of transgenic IVF embryos by vesicle microinjection to generate transgenic blastomeres to be used as donor cells for cloning. A high efficiency in mosaicism reversal and multiplication of transgenic embryos was attaineded [3].

The separation of transgenic blastomeres followed by the aggregation of two-cell fused embryos or by the asynchronous younger blastomere successfully multiplied transgenic embryos, and theoretically reduces mosaicism rates in future offspring [15]. This technology can also be used to multiply embryos from animals with high genetic value.

Another technique that we assessed was reducing mosaicism through gamete cloning. We demonstrated that a sperm and oocyte can be efficiently cloned [47,45,49]. Green haploid androgenic blastomeres produced with the injection of a single sperm by *egfp* ICSI-MGT could be used to fertilized oocytes resulting in several homogeneous expressing embryos. 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 [49] 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.

The applications of these technologies are subject to our capacity for imagination and innovation. Animals that we have generated with some of these methodologies (Figure 1, 2 and 3) have formed a kind of postcard into the future that demonstrates the possibility to use these technologies in our countries.

IX. CONCLUSIONS

Significant advancements have been made in the course of the recent years in micromanipulation and transgenesis techniques. In our lab we have been

evaluating ICSI and Nuclear transfer mainly to produce transgenic embryos. We used also transgenesis developed to mark with flurorecence protein expression and develop new micromanipulation technique in domestic animals like gamete cloning. 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.

Figure 1. BS Ñandubay Bicentenario cloned horse produce in 2010.

Figure 2. Pampa dynasty: clones produced from a Growth Hormone transfected cell line in 2002.

Figure 3. Esperanza lamb generated by ICSI produced in 2008.

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