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Transgenesis in farm animals

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Abstract

The production of transgenic animals has many biotechnological applications; however, available techniques still remain inefficient. This review summarizes the contributions of our group to transgenesis in domestic animal. One of the most traditional transgenesis techniques in farm animals is somatic cell nuclear transfer using genetically modified somatic cells. Using this technique, we produced transgenic cloned cows capable of producing human growth hormone in the milk, and viable offspring was obtained after recloning of these transgenic animals. Another technique available to produce transgenic animals is intracytoplasmic sperm injection (ICSI). With this technique, we could obtain transgenic embryos in five different domestic species (bovine, ovine, feline, porcine and equine). To improve transgenic ICSI (ICSI-Tg) efficiency in cattle, different chemical activation treatments were evaluated, including ionomycin followed by 6-Dimethylaminopurine (6-DMAP), strontium chloride and ethanol. In addition, in the latest years, our group has developed several alternative methods to produce transgenic animals involving injection of ooplasmic vesicles, liposomes or cells previously incubated with the transgene, into zygotes or enucleated oocytes respectively. All of these treatments proved to be efficient to induce the expression of exogenous DNA into pre-implantatory embryos. However, high levels of transgene expression mosaicism were detected. For that reason, different approaches were also tested to reverse mosaicism, including a novel cloning gamete technique. We conclude this review with a brief description of novel molecular tools for transgenesis, including enzymes such as transposases, Zinc finger and Talem nucleases, which could help to increase transgenesis efficiency in domestic species. The new transgenesis variants described in this review aim to turn animal transgenesis accessible to a greater number of research groups.

Keywords: gene transfer, ICSI-Tg, micromanipulation, transgenesis.

Introduction

Transgenic animals are important tools for basic and applied biotechnology. These genetically modified animals are produced through the introduction of an exogenous gene into the animal genome. Only the integrated transgenes will be transmitted to the next generations as mendel traits. This revision analyzes available methods to obtain transgenic animals. The main objective of this paper is to present, technique by technique, promising methods recently developed or evaluated in our laboratory.

Gene transfer by pronuclear microinjection

The first method introduced for transgenic animal production was the microinjection of exogenous DNA into the male pronucleus of zygotes (Gordon *et al*., 1980). Transgenesis by microinjection is still used to generate transgenic mice. This procedure has also proven to be useful in species such as rabbits, sheep, pigs (Hammer *et al*., 1985) and goat (Freitas *et al*., 2007), but it is dependent on a proper visualization of the male pronucleus. Besides, in some species, such as cattle, it has a very low efficiency (Eyestone, 1999). In the original work of Gordon *et al*. (1980), it was reported that transgenesis could also be achieved to a lesser extent by cytoplasmic injection of the transgene. We developed strategies to increase the efficiencies reported by these authors for cytoplasmic injection of transgene, in order to apply this technique to species for which pronuclear microinjection shows low efficiency. This will be described on the section Gene transfer by cytoplasmic microinjection.

Transgenesis by somatic cell nuclear transfer

Cloning by somatic cell nuclear transfer (SCNT) has allowed the production of elite offspring and the generation of transgenic animals for agricultural or biomedical purposes. Several factors influence the results of SCNT, including enucleation methods (Moro *et al*., 2010), fusion parameters, activation procedures (Vichera *et al*., 2009, Canel *et al*., 2010), donor cell lines (Salamone, 2006) and also the degree of cell cycle synchrony between the donor cell and the recipient oocyte (Wells *et al*., 2003). One of the main problems of nuclear transfer technique is the low survival rates of cloned embryos and fetuses (Kishigami *et al*., 2008). It is well known that chemical activation procedure is one of the key factors affecting fetuses' viability (Ross *et al*., 2009). For that reason, we evaluated the combination of ionomycin with a compound isolated in Argentina, Dehydroleucodine (DHL), for chemical activation of both ICSI and SCNT (Vichera *et al*., 2009). Although DHL did not result in higher embryo development rates than 6-Dimethylaminopurine (6-DMAP), Canel *et al*.

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(2010) showed that DHL induced pronuclear formation dynamics similar to *in vitro* fertilization (IVF) and lower polyploidy rates than drugs frequently employed to induce chemical activation.

Despite SCNT drawbacks, its main advantage is the possibility to employ genetically modified somatic cells for the production of cloned transgenic animals (Schnieke *et al*., 1997, Cibelli *et al*., 1998). In this way, in September 2002, Salamone *et al*. produced the first cow of South America capable of expressing human growth hormone (hGH) in the milk. Transgenic cows can be employed as bioreactors and it was estimated that only 15 animals would be necessary to meet the current global needs of this protein (Salamone *et al*., 2006). However, production of cloned transgenic animals by SCNT is expensive and generally shows a low efficiency. In addition to problems related to the cloning procedure, there are several additional disadvantages for transgenic cloning. Between transgenic cell lines problems, we observed that different transfection events of the same somatic cell line resulted in unequal fetal survival rates. An experiment developed with the company Biosidus, with a fetal cell line derived from a 75 days female Jersey-bred fetus, transfected with a transgene on three different occasions using the same protocol, resulted in births only from two of the three transfection events of the same cell line. This shows that the transfection event provides an additional source of variability in the production of viable transgenic animals. It is very important to produce well characterized transgene integration and gene expression. However, after non homologous transgene integration, a heterogeneous number of transgene copies is introduced at different chromosomes locations. Therefore, clonal propagation of transfected cells is the only way to characterize the transgene integration status. Anyway, it must be considered that this process can compromise the viability of donor cells. Another strategy to increase the homogeneity among transgenic animals is to reclone the first generation of transgenic calves. Recloned calves were obtained in an experiment that consisted on the evaluation of survival rates after a second round of cloning using transgenic fibroblasts obtained from the umbilical cord or the ear of the cloned calves. Seven births were obtained from the original fetal cell line, one birth was obtained from the recloned umbilical cord and two calves were obtained from the recloned ear fibroblasts. Although lower blastocyst rates were obtained after recloning, this technique provided an additional method to obtain transgenic animals.

ICSI mediated gene transfer (ICSI-tg)

Several authors reported alternative techniques to SCNT and pronuclear microinjection, involving sperm cells as transgene vectors. These techniques are based on the common feature that the spermatozoa carry the transgene, but they differ in the mechanism employed to deliver the sperm into the oocyte. In this way, the variants of this technique consist of sperm co-incubation with the transgene followed by laparoscopic insemination (LI; Lavitrano *et al*., 2002), IVF (Lavitrano *et al*., 1989) or ICSI (Perry *et al*., 1999). Despite LI and IVF are more efficient in terms of embryo production, the transgenesis results obtained after these two procedures are controversial (Brinster *et al*., 1989; Lavitrano *et al*., 1989). ICSI-Tg on the contrary, was repeatable and effective to produce mice offspring (Perry *et al*., 1999) and pigs (Kurome *et al*., 2007). In our experience, despite obtaining high rates of morulae/blastocysts with LI and IVF techniques, none of the embryos expressed the transgene (Pereyra-Bonnet *et al*., 2010). In contrast, 91.6% *egfp* expressing morulae and blastocysts were obtained by ICSI-Tg (Pereyra-Bonnet *et al*., 2011).

We used ICSI-tg assisted by chemical activation in five different mammal species including sheep, pig, cat, cattle and horse, and observed that this technique could easily adapt to all these species. Sperm cells were coincubated with pCX-EGFP plasmid and then injected into MII oocytes. The chemical activation protocol consisted of ionomycin immediately after injection, a 3 h window to allow the second polar body extrusion and finally, 6-DMAP treatment. High *egfp* expression rates were detected in the five species (23 to 60%) on the fourth day, and green blastocysts were obtained in cattle, sheep and cat (Pereyra-Bonnet *et al*., 2008).

In cattle, ICSI-Tg had not been reported prior to our report (Pereyra-Bonnet *et al*., 2008). The main reason for the failure of previous attempts in ICSI-Tg is the poor embryo development results obtained after conventional ICSI in this species. For that reason, in a further study we determined the best conditions for ICSI-Tg in cattle and evaluated five activation treatments to assist the ICSI. In this case, after injection of sperm coincubated with transgene into metaphase II oocytes, chemical activation procedure consisted on an initial treatment with ionomycin (Io), followed by activation with: DMAP (Io-DMAP), an additional treatment of Io immediately before DMAP (2Io-DMAP), only a second treatment of Io (2Io), ethanol (Io-EtOH) or strontium chloride (Io-$SrCl_2$). Fertilization rates did not differ significantly between treatments. All EGFP positive embryos (100%) had been fertilized, while at least 60% of non transgenic embryos still showed a condensed sperm head by day 4. Blastocysts rates after 2Io-DMAP tended to be higher than the other treatments. Most remarkably, ICSI-Tg in cattle, in these conditions, resulted in EGFP expressing blastocysts rates of over 80% (Bevacqua *et al*., 2010).

The main drawback of ICSI-Tg is the high mosaicism rates of embryos, comparable to observed for pronuclear microinjection technique. One possible explanation for mosaicism is that the transgene integrates into the embryo genome after the first cell division (Perry

et al., 1999; Szczygiel *et al*., 2003; Kaneko *et al*., 2005; Smith and Spadafora, 2005). This could be due to the persistence of extrachromosomal molecules of transgene during subsequent mitotic divisions, which integrate later on development (Celebi *et al*., 2002).

Gene transfer by cytoplasmic microinjection

After our ICSI experiments, we decided to evaluate if other cells were also capable of transferring the transgene. We demonstrated that not only the sperm but also other cell types, cell fragments or liposomes could transfer the exogenous DNA to the embryo nucleus. When cumulus cells, cell fragments (which we called ooplasmic vesicles or vesicles), or liposomes were incubated with transgene, when just the plasmid containing the transgene was injected into the cytoplasm of MII oocytes, transgene expressing embryos were obtained after IVF and parthenogenic activation (Vichera *et al*., 2010; Pereyra-Bonnet *et al*., 2011, Bevacqua *et al*., 2012). Using confocal microscopy, we observed that the transgene is adhered to cells and to ooplasmic vesicles. These approaches could greatly simplify available transgenesis techniques.

Despite the many advantages showed for these techniques, high transgene expression mosaicism rates were detected in embryos. As this problem is also observed after pronuclear microinjection and ICSI-Tg, we decided to evaluate different alternatives to prevent or diminish mosaic transgene expression patterns. Two of them also involve cloning and will be described in the next section. The other, requires incubation of IVF zygotes on cell cycle inhibitors (DMAP and DHL) during the first pronuclear phase, with the aim of lengthening the first cell cycle and assuring transgene integration at early times. This experiment showed that the cell cycle inhibitor DMAP induced a greater phosphorylated histone H2AX foci area (which acts as indicator of double stranded DNA breaks), and tended to reduce mosaic transgene expression patterns (Bevacqua *et al*., 2012).

Different approaches to embryo multiplication and mosaicism reversion

One strategy employed to reverse mosaicism consisted of the production of transgenic embryos by vesicles microinjection followed by cloning of day 3 transgenic blastomeres. Mosaicism was reversed in this way and multiplication of transgenic embryos was also achieved (Bevacqua *et al*., 2012).

Another strategy evaluated for mosaicism reversion was embryo aggregation. This technique consists of the disaggregation of day 3 embryos, the selection of the transgenic blastomeres and the aggregation of each one (Vajta *et al*., 2000) with two fused embryos (putative tetraploid embryos) in the WOW system. The earliest blastomeres tend to give rise to trophoblast cells and the transgenic one gives rise to the embryo, and localizes to the ICM. In this way, this technique allows transgenic embryos multiplication and, theoretically, it reduces the rate of mosaicism of the future offspring (Hiriart *et al*., 2010). This methodology can be used to multiply high genetic value embryos, by means of embryo separation into their blastomeres and blastomeres subsequent aggregation with future trophoblast cells generated from poor value embryos.

Another option to reduce mosaicism is gametes cloning. We demonstrated that sperm and oocytes can be efficiently cloned (Vichera *et al*., 2011a, b), obtaining several copies of one sperm or oocyte nucleus. Androgenic *egfp* expressing haploid blastomeres were produced by injecting a single sperm that was previously incubated with pCX-EGFP into an enucleated oocyte. Then, the green androgenetic blastomeres obtained were fused to mature oocytes in order to "fertilize" them, and in this way, several biparental embryos with uniform transgene expression were obtained. In addition, this innovative technique allows the determination of sperm nucleus sex prior to biparental embryo reconstruction.

Oocyte genome cloning is also a method to generate homogeneous transgene-expressing embryos (Vichera *et al*., 2011a). Firstly, haploid maternal embryos were generated by parthenogenesis obtaining several blastomeres that are considered as a clone of the original gamete. To generate *egfp*-expressing blastomeres, activated oocytes were injected with liposomes containing pCX-EGFP. These green blastomeres were then used to generate biparental embryos with homogeneous transgene expression.

The possibility to clone spermatozoa or oocytes genomes opens the possibility to achieve haploid cell lines, which could have potential to generate an unlimited number of two-parent embryos through combination of these haploid cells with haploid hemizygotes cells of the opposite sex.

New tools to increase the integration

Results presented so far clearly indicate that many techniques are capable of transferring the transgenes to the cytoplasm and also to the nuclei of the oocytes or presumptive zygotes. However, transgene arrival to the nuclei does not imply its subsequent integration into the host genome. These observations make necessary the development of techniques to facilitate the integration of the transgene.

To this aim, active transgenesis techniques, that involve the engineering of the plasmid constructs to interact with specific enzymes, were developed. One of the first active transgenesis techniques introduced was lentivirus mediated transgenesis (Lois *et al*., 2002). Despite having high efficiency, this technique shows several disadvantages, including high embryonic lethality rates, relatively small size of the transgene

(9.5 kb) that can be carried by the vector, and requirement of special biosafety facilities to work with viruses. For that reason, other active transgenesis techniques were developed. Recently, recombinases, integrases and meganucleases derived from lower organisms proved to be active in mammalian cells, and that allowed the development of new transgenesis strategies involving active enzymes with lower risk than lentiviruses but equivalent effectiveness. In this respect, our group introduced meganucleases mediated transgenesis, achieving increased integration efficiency regarding to cytoplasmic transgene injection in the absence of the enzyme (Bevacqua *et al*., 2011). Nowadays, we are waiting results regarding the efficiency of this technique to produce transgenic sheeps. Other interesting nucleases recently developed are Designed meganucleases (Zinc Finger nucleases and TALEN). These nucleases carry two domains, a DNA binding domain linked to a nonspecific DNA cleavage domain (Kim *et al*., 1996; Cermak *et al*., 2011). The major advantage of these techniques is that recognition specificity of arbitrarily chosen chromosomal sites can be controlled, without prior manipulation of the genome (Bibikova *et al*., 2003). However, accessibility to these nucleases is limited to only a few laboratories. Transposon-mediated transgenesis is another approach recently tested. It already proved to be efficient for the production of transgenic animals, including pigs (Garrels *et al*., 2011).

Conclusion

The material presented in this review shows that there are many new alternatives available to transfer transgenes to host cell nucleus, which combined with new molecular tools, could result in increased integration efficiencies. The proper combination of these available tools will allow a greater number of research groups to gain accessibility to animal transgenesis.

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