Somatic cell nuclear transfer: Where it stands
Transferencia de células somáticas nucleares: Donde estamos?

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Abstract

Animal cloning by nuclear transfer is a technique that has grown immensely during the past decade. Scientists have worked arduously to better understand the processes involved in embryo and fetal development in order to improve the efficiency and results of the procedure. Although many different animal species like rabbits, mice, sheep, goats, horses and pigs among others have been used for these purposes, bovines have been worked with to a greater extent. Nuclear transfer has aided in the creation of models that allow the study of cellular processes such as gene function, genomic imprinting and reprogramming, as well as genetic diseases, gene therapy, cancer and the regulation of development. In spite of all the efforts that have been made, the current efficiency of the technique is well bellow standards that would make it viable for commercial uses. The application of this biotechnology in the fields of animal production and biomedicine are infinite, ranging from cloning elite farm animals for dissemination of superior genetics and protection of endangered species, to the creation of mammalian bioreactors for production of nutrients, pharmaceutical proteins and substances that may help treat human diseases. Transgenic farm animals are now a reality that allows the selection of specific genes that express valuable characteristics for production or resistance traits. Somatic cell cloning is a technique in which the nucleus of a somatic cell is transferred into an enucleated metaphase II oocyte in order to generate a new individual that has an identical genetic composition as the cell from which it was produced. After the successful cloning of "Dolly", the sheep, it was demonstrated that a process called nuclear reprogramming (reversion of a differentiated nucleus back to totipotent status), could completely reactivate genes that had been inactivated during tissue differentiation. Even though many breakthroughs have been made during the development of the technique there are still many processes that are poorly understood and that lead to abnormalities in the cloned animals which are commonly fatal or prevent their normal development. This is why it becomes necessary to research the source of the medical problems associated with cloned animals and develop effective treatments that help reduce neonatal and post-birth morbidity and mortality rates, as well as pregnancy loses that are specially common at the beginning of fetal development. Now is the time to strive to better understand the difficulties associated with this technique in order to offer mankind an alternative that can contribute to the solution of world hunger and disease.

Key Words

Somatic cell, nuclear transfer, clone, embryo, oocyte

Resumen

La clonación de animales mediante transferencia nuclear es una técnica que ha crecido inmensamente durante la última década. Los investigadores han trabajado arduamente para comprender mejor los procesos involucrados en el desarrollo embrionario y fetal y así mejorar la eficiencia y resultados obtenidos con el uso del procedimiento. Aunque son varias las diferentes especies animales, como conejos, ratones, ovejas, cabras, caballos y cerdos entre otros, que han sido utilizadas para estos propósitos, se ha trabajado en mayor medida con los bovinos. La transferencia nuclear ha ayudado en la creación de modelos que permiten el estudio de procesos celulares tales como la función de los genes, el imprinting y reprogramación genómica, al igual que patologías genéticas, terapia genética, el cáncer y la regulación del desarrollo. A pesar de todos lo esfuerzos que se han hecho, la eficiencia que se obtiene actualmente con el uso de la técnica esta muy por debajo de los estándares que la harían

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viable para utilizarla comercialmente. Las aplicaciones de esta tecnología en la áreas de producción animal y biomedicina son infinitas, desde la clonación de animales domésticos de elite para la diseminación de genéticas superiores y la protección de especies en peligro, hasta la creación de biorreactores mamíferos para la producción de nutrientes, proteínas farmacéuticas y sustancias que pueden ayudar en el tratamiento de patologías humanas. Los animales domésticos transgénicos son ahora una realidad que permite la selección de genes específicos que expresan características de producción valiosas o rasgos de resistencia. La clonación de células somáticas es una técnica en la cual el núcleo de una célula somática es transferido dentro de un oocito enucleado en metafase II, con el fin de generar un nuevo individuo que posee una composición genética idéntica a la de la célula de la cual fue producida. Después de la clonación exitosa de la oveja "Dolly", quedo demostrado que un proceso llamado reprogramación nuclear (reversión de un núcleo diferenciado devuelta a un estatus totí potencial), podía reactivar completamente genes que habían sido desactivados durante la diferenciación tisular. A pesar de que se han obtenido muchos descubrimientos durante el desarrollo de la técnica, aun hay muchos procesos que son pobremente comprendidos y que generan anormalidades en los animales clonados las cuales son comúnmente fatales o impiden su normal desarrollo. Por esto se hace necesario investigar la fuente de los problemas médicos asociados a los animales clonados y el desarrollo de tratamientos eficientes que ayuden a reducir las tasas de morbilidad y mortalidad neonatal y pos-nacimiento, al igual que las perdidas durante la preñez que son especialmente comunes al comienzo del desarrollo fetal. Este es el momento de hacer un gran esfuerzo para comprender mejor las dificultades asociadas a esta técnica con el fin de ofrecer a la humanidad una alternativa que pueda contribuir a la solución del hambre y las enfermedades a nivel mundial.

Palabras clave

Célula somática, transferencia nuclear, clonación, embrión, oocito.

Introducción

The arrival of "Dolly", the cloned sheep, came as surprise to scientist and common people alike, even thought animal cloning had benefitted from decades of intensive research. Despite heroic efforts made throughout the 50s, 60s and 70s to understand the fundamental mechanisms involved in nuclear reprogramming, scientist were actually "blinded" by the inability to obtain live offspring from amphibian cells [52]. Renewed hope came when lambs were obtained after nuclear transfers from embryonic blastomeres [98], and inner-cell-mass cells [72]. Several other domestic and laboratory animals were cloned successfully, which confirmed the possibility of producing embryo derived cloned offspring and lead to the establishment of private companies whose aim was to improve and apply nuclear transfer technology to cattle breeding. In spite of it all, the application of embryo technology in a commercial setting failed because of two directly related factors. First off was the low efficiency of embryo multiplication due to poor development rates up to blastocyst stage. The inefficiency lies in many areas, such as the donor cell types, cell cycle stages, genetic background of donor cells and recipient oocyte, nuclear transfer procedure, and culture environments [16, 29, 62]. At the moment, the efficiency for nuclear transfer is between 0–10%, which means 0-10 pregnancies for every 100 cloned embryos. Second, was the association between high embryo mortality throughout gestation, production of oversized calves, extended gestation length, and increased neonatal morbidity and mortality with pregnancies derived from nuclear transfer embryos. Developmental defects, including abnormalities in cloned fetuses and placentas, in addition to high rates of pregnancy loss and neonatal death have been encountered by every research team studying somatic cell cloning [78]. It has been proposed that low cloning efficiency may be largely attributed to the incomplete reprogramming of epigenetic signals [6, 14, 36, 63].

A renewed interest in nuclear transfer has stemmed from the successful use of donor nuclei from fetus-derived cells that had a novel gene incorporated into their chromosomes (transfected) to produce transgenic farm animals [66, 38, 11]. However, regardless of the potential benefits of producing transgenic farm animals, problems associated with loss during gestation and neonatal mortality have increased substantially with the use of somatic cells in nuclear transfer [31]. Regardless of the inefficiencies of this process currently, morphologically

normal living animals have been produced in 10 species during the past few years including sheep [99], mouse [83], cow [22], goat [1], pig [61], rabbit [10], cat [68], mule [102], horse [23], and rat [105].

Somatic cell cloning (cloning or nuclear transfer) is a technique in which the nucleus (DNA) of a somatic cell is transferred into an enucleated metaphase-II oocyte for the generation of a new individual, genetically identical to the somatic cell donor [78]. After the successful cloning of "Dolly", the sheep, it was demonstrated that a process called nuclear reprogramming (reversion of a differentiated nucleus back to totipotent status), could completely reactivate genes that had been inactivated during tissue differentiation. Nuclear cell transfer may be used to preserve endangered species, production of transgenic animals for pharmaceutical protein production or xeno-tranplantation, or generating various copies of elite farm animals. In the future, when the process becomes much more efficient, it will offer an enormous amount of biomedical possibilities in therapeutic cloning and allo-transplantation. Furthermore, cloning has become an essential tool for gene function studies [9], genomic re-programming [15, 54, 76, 101], genomic imprinting [73], regulation of development, genetic diseases, gene therapy and even cancer.

Somatic cell nuclear transfer is much more widely and efficiently practiced in cattle than any other species, making this arguably the most important mammal that has been cloned to date [57]. One would think that the laboratory mouse with its well characterized and easily manipulated genome, precisely described embryonic development, short gestation period and large litter size would be the best model for the type of studies mentioned above, but the reality is that mice are very difficult to clone. First reported in 1998 [83], the successful cloning of viable mice from somatic cells is only achieved by a reduced number of laboratories worldwide. On the other hand, cattle somatic cell nuclear transfer, has about 3 - 5 fold higher average efficiencies and is practiced on a more regular basis. After years of practice, laboratory mouse cloning laboratories cannot achieve the 10-20% cloning efficiency obtained by many cattle cloning labs. Oback and Wells (2007) published that of 160 laboratories in 37 nations, 80 of them in 24 nations were cloning cattle, what would account for half of all cloning organizations worldwide. In light of this information, this paper will focus primarily and reference information on cattle cloning, with some views on other mammalian species.

Methodology

The complexity of the nuclear transfer procedure makes it impossible to standardize all experimental details across the different research groups [57] working in the field. The technical differences that result from the use of different protocols, lead to the fact that two procedures are not exactly the same. Three separate types of protocols can be distinguished and are mainly differentiated by the method of enucleation (removal of genetic material in order to obtain a cytoplast), whether the genetic material is surrounded or not by a nuclear envelope.

Conventional zona-intact nuclear transfer, consist of aspirating the maternal chromosome and surrounding cytoplasm in a small plasma membrane envelope [57]. This method is the most popular has been used for more than 20 years.

Zona-free nuclear transfer is a simplified variation of the first method that doubles the amount of structures that can be worked with and by doing so effectively doubles the cloned offspring production, due to the ease of operation a reproducibility that it offers. This method is easier to learn for beginners with no previous micro-manipulation experience [57]

Hand-made cloning is the most radical of the procedures and consists of manually bisecting the zona-free oocyte with a micro-blade, and then discarding the chromatin-containing half. Two enucleated demi-cytoplasts are then fused to reconstitute the original volume before the nuclear transfer [57]

Cellular differentiation is a highly regulated and poorly understood process, in which cells specialize in performing specific functions and loose their ability to perform others. It appears to be linked to a successive restriction in chromatin accessibility and consequently reduced number of expressed genes[44,53]. Dedifferentiation is the reverse process, which occurs in mammals during regeneration or carcinogenesis, both of which are relatively rare processes. A hypothesis has been emerging that the donor cell differentiation status is inversely correlated to the cloning efficiency [56, 35]. If this were true, somatic cells would be one of the most difficult cell types to clone and their efficiency would be much lower than that of an embryonic or germ cell.

Nuclear reprogramming is the term used to describe the ability that the oocyte cytoplasm has to override any transcriptional programme that is present in the donor

cell [95]. In other words, it is the capacity that the oocyte cytoplasm has to develop a reconstructed embryo into a blastocyst or even a viable animal, instead of developing into a population of fully differentiated cells, like the one used as nuclear donor. Although the reason for this molecular dominance of the oocyte is unknown, there are two hypotheses that try to explain it. It could simply be a matter of volume, seeing as the oocyte is 125 times larger and therefore would contain a four-thousand fold more oocyte-specific factors. On the other hand, the reason could be based on the quality, and not quantity, of those oocyte-specific factors whose natural function is to reprogram the incoming sperm genome after fertilization [57].

Technical Procedure

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above, but the reality is that mice are very difficult to clone. First reported in 1998 [83], the successful cloning of viable mice from somatic cells is only achieved by a reduced number of laboratories worldwide. On the other hand, cattle somatic cell nuclear transfer, has about 3 - 5 fold higher average efficiencies and is practiced on a more regular basis. After years of practice, laboratory mouse cloning laboratories cannot achieve the 10-20% cloning efficiency obtained by many cattle cloning labs. Oback and Wells (2007) published that of 160 laboratories in 37 nations, 80 of them in 24 nations were cloning cattle, what would account for half of all cloning organizations worldwide. In light of this information, this paper will focus primarily and reference information on cattle cloning, with some views on other mammalian species.

Selection of Donor Cell

The process of cloning animals is separated into several steps and involves the use of specialized equipment by highly trained personnel. The first step consists of finding a suitable cell to be the nuclear donor, from which to clone from. These donor cells (primary cells) can be taken directly from the animal, but they are usually propagated in vitro in order to facilitate storage and manipulation. Due to the fact that they usually produce stable dividing and homogeneous primary lines, have a reasonably long life span and can be frozen and thawed with limited viability loss, fibroblasts deriving from skin are often used as nuclear donor cells. On the other hand, many somatic cell types including mammary epithelial cells, ovarian cumulus cells, fibroblast cells from skin and internal organs, various internal organ cells, Sertoli cells [58, 85], macrophage [85], and blood leukocytes [22, 32], have been used with success in nuclear transfer procedures. Forsberg et al. (2002), conducted large numbers of embryo transfer in cattle using different somatic cell types as donors. In his experiments, cumulus cells gave an overall 15.2% calving rate, fetal genital ridge cells, and fibroblast cells produced a 9% calving rate, while adult fibroblast cells gave the lowest calving rate of only 5%. It could be said that in general, embryos cloned from fetal cells produced higher pregnancy and calving rates than those from adult cells [78]. Other studies have shown that fetus cells and adult cells lead to comparable blastocysts development rates. Cloning by using skin cells offers the advantage of easy accessibility and noninvasiveness without animal sex or age limitations. Previously, successful cloning of adult animals has largely been limited to the use of female reproductive system cells [46].

Cloned embryos, fetuses and offspring have been proofed to suffer from epigenetic abnormalities, such as aberrant gene expression [2, 5, 33, 60, 103], DNA-methylation [6, 14, 36, 37], and histone-methylation [65]. Some of the symptoms suffered by these clones must be of epigenetic nature since they are not transmitted from parent to offspring [67], and it is still unknown to what degree, presumably non-reprogrammable, genetic factors contribute to the death of failing clones. In light of this, donor cell choice should be thorough to avoid influencing potential genetic problems in clones.

It has become evident that chromosomal anomalies derived from the nuclear donor can affect cloning success. It is therefore important to have a rigorous pre-screening process of the donor cells before the nuclear transfer. Spontaneous mutations arising during ageing and/or time in vitro could affect cloning efficiencies, however, cells after long-term culture [46], or near the end of their replicative life span have no significantly reduced cloning efficiencies [47]. Another form of genetic damage is the progressive shortening of telomeres as a consequence of cell division in the absence of telomerase activity, known as telomere erosion. Depending on the cell type and time of culture in vitro, there are differences in the telomere length of the donor cell. Since senescent cultured bovine fibroblasts with shortened telomeres can be successfully used as donors [47], it can be inferred that telomere length adjustment and telomerase reactivation can be ascertained after nuclear transfer. This is why it is continuously and consistently demonstrated that cellular senescence occurring in vivo does not correlate in any way with ageing of the organism, and therefore rebates the popular belief that donor cell choice could result in cloned offspring suffering from premature ageing or having a reduced life span.

Post-implantation development and post-natal viability improve dramatically when F1 cells are used [17, 64, 84, 85]. All genetic loci in F1 animals have maximum expression of heterozygosis, more so if they come from two pure (inbreed) strains. This is a well known fact in farm animal breeding referred to as hybrid vigor. In the practice, the genetic background of donor cells will be dictated by the application in which it will be used, like in progeny-tested bulls. Never the less, it will be necessary to first identify a favorable genotype, which up till now has not been found.

Recipient Oocyte

After comparing oocytes in different developmental stages and experimenting with different maturation treatments, scientist have been able to define a subpopulation that produces the best results in cattle somatic cell nuclear transfer. This subpopulation consists of non-activated MII oocytes from developmentally competent follicles of slaughtered adult animals [57]. Maturing oocytes from germinal vesicle to MII stage can be done in vivo or in vitro, as both types have been used in successful cloning procedures. As described previously with donor cells, hybrid vigor of F1 derived oocytes tends to be beneficial for the development of cloned embryos [85, 26].

The first process that the recipient oocyte has to undergo, after it has been removed from the follicle and selected for presenting excellent morphology, is maturation. Maturation consists of leading the oocyte up until the second meiotic division in the metaphase stage, also known as metaphase II. The procedure may be done in vivo (preferred for laboratory animals), or in vitro (preferred for large domestic animals). Maturation is usually performed using complex mediums, generally TCM-199®, supplemented with growth factors, hormones and bovine fetal serum that are then kept in incubators that control CO2/O2, and a temperature that best suits the species of interest. The time required for the oocytes to complete maturation varies with the species, but for ruminants it generally falls between 18-24 hours.

Before performing the nuclear transfer, the recipient oocyte DNA must be removed or destroyed in a way that the viability and reprogramming potential of the cytoplast will not be compromised. This can be done by any of three protocols described earlier in this paper (methodology). Tecirlioglu et al. (2005), compared gentle aspiration with a glass needle (zona-free nuclear transfer protocol), versus oocyte bisection (hand-made cloning), and found that the use of either protocol resulted in similar cloning efficiencies. Other alternatives like chemically-assisted enucleation, in which a combination of microtubule- depolimerazing and oocyte-activating drugs are used, or physically destroying the chromatin with the use of X-ray irradiation are being studied, but are yet to prove in vivo results.

Prior to enucleation, oocytes must be stripped naked of the cumulus cells by submerging them in a buffered solution (PBS or TCM-199® + HEPES), containing hialuronidase (1-2 mg/mL), and aspirating them over and over inside a pipette point tip. After this has been accomplished, the oocytes are carefully selected based on morphological quality and presence of the first polar body (polar corpuscle), indicator of the metaphase II stage. The extrusion of the polar corpuscle also serves as a reference point for finding the chromatin that has to be removed, because it usually lies at the borders of the oocyte close to the polar body. Once this has been done the oocyte is exposed to cytoskeleton un-stabilizing substance, called citochalasin, in order prevent its rupture or destruction during the procedure. This substance destabilizes the actine filaments in the cellular membrane, and by doing so makes the oocyte more elastic. At this point, the remaining oocytes are ready to be enucleated.

In most domestic species it is impossible to see uncolored chromatin inside the oocyte. For this reason, special staining substances like DNA specific fluorocrome are used to visualize the genetic material. The oocyte is then exposed to ultraviolet light, for the least amount of time possible (seconds), in order to locate the material that will be removed. UV-light has a devastating effect on the oocyte and seriously compromises its viability if maintained for long periods of time. The effect of ultraviolet light UVC (254 nm) and UVA (>330 nm) has been studied on bovine oocytes at the germinal vesicle and metaphase II stage. Both UVA and UVC irradiation caused abnormalities of meiosis and production of maturation promoting factor (MPF) at both germinal vesicle and metaphase II stages. This resulted in abnormal parthenogenetic activity, with loss of the female pronucleus being seen after UVC irradiation and an abnormal female pronucleus after UVA irradiation in metaphase II oocytes [18].Other studies of brief exposure to UV light of bovine secondary oocytes revealed increased membrane lysis and increased methionine uptake, but reduced methionine incorporation into protein and a marked difference in the patterns of protein synthesis [69].

Nuclear Transfer

When using somatic cells as nuclear transfer donors, they are usually put into a tripsine:EDTA solution (0,1-0,25% tripsine for 0,02% EDTA), in order to individualize them. Generally, in vitro cultures are established so that the cells can be maintained. If this is the case, the cells are taken from the culture medium and washed twice with a buffered saline phosphate solution (PBS), without calcium or magnesium. Afterwards they are kept for 1-2 minutes in the tripsine:EDTA solution and then culture medium containing 10% bovine fetal serum is

added in order to inactivate the tripsine. Finally, cells are suspended, centrifuged and re-suspended in culture medium. Different types of somatic cells have been used successfully to clone animals, yet it has not been possible to establish which type is most appropriate for this purpose.

The two principal methods by which the genetic material of a donor cell is transferred into the recipient oocyte are, whole cell nuclear transfer followed by cellular fusion, or microinjection of isolated nuclei. Both methods have been used successfully and the differences between them have not been critical. Direct comparisons between the two methods in cattle have found no significant differences in calving rates [24]. Plasma membrane fusion can be induced by several different methods such as the use of polietilienoglicol, inactivated Sendai virus, liposomes (lipidic vesicles), or electrical impulses (electrofusion or electroporation), the most commonly used and preferred method for the majority of animal species.

Electro-fusion has to be performed in a solution with low electrical conductance in order to avoid the production and dispersion of heat. Most laboratories use a manitol solution containing 0,28-0,3M of manitol; 100µM of MgSO4; 50µM of CaCl2; 0,01 mg/mL of BSA; pH 7,2 [4]. The procedure usually involves one impulse of alternate current and one or more impulses of continuous current. The alternate current polymerizes the donor cell and recipient oocyte cytoplasm parallel to de electrodes. The direct current induces the formation of pores in the cellular membranes that cause fusion between the cells. In order to obtain high fusion rates, there must be strong contact between the donor cell and recipient oocyte and perfect parallel alignment with the electrodes. The duration, intensity and number of pulses vary according to the species and equipment being used. Generally, continuous current is applied at a voltage of 1,5 Kv and duration of 60-100 µs.

The microinjection procedure consists of aspirating a cell into a pipette, whose diameter is less than that of the cell, which will cause rupture of the cellular membrane. The same pipette will be used to inject the nucleus into the cytoplasm of the recipient oocyte. Another option when introducing nuclei into oocytes is to perforate the membrane and deliver the nuclei directly into the ooplasm by using a piezo-controlled pipette holder [83]. Studies using pigs have shown that cells can be injected intact and this way the process may be simplified, but the efficiency of this procedure is yet to be tested in other domestic species.

Artificial Activation

Cloning, replaces physiological activation of the oocyte that occurs during fertilization and for which the spermatozoid is responsible. Since mammalian donor cells are unable to activate the recipient cytoplast, various artificial activation protocols have been employed to mimic the sperm-induced cellular events typically occurring during oocyte activation [57]. Flaws during oocyte activation can compromise the integrity of the transplanted chromatin. Furthermore, timing of the activation procedure can affect nuclear reprogramming.

Activation refers to inducing the degradation of enzymatic complexes responsible for maintaining the oocyte in metaphase II stage. By doing so, it allows the completion of meiosis leading to the beginning of embryonic development. Matured oocytes stay blocked in metaphase II stage by the action of a protein complex called M phase promoting intracellular factor (MPF). MPF activates by phosphorilation other proteins that are responsible, among other things, for chromatin condensation and keeping the oocyte blocked in metaphase II stage. MPF maintains the metaphase arrest by keeping the chromatin in a condensed state and stabilizing the meiotic spindle [80]. High MPF activity is sustained by another activity called the cytostatic factor (CSF). CSF inhibits the anaphase promoting complex (APC), thereby preventing the metaphase–anaphase transition [79]. During fertilization, the spermatozoid that penetrates the oocyte is responsible for the signal that activates MPF degradation, triggering the final steps of meiosis and the start of embryonic development. This process of activation inside the oocyte depends on the release of several calcium waves. Based on this principle, different chemical and physical procedures have been established in order to induce activation either by having extracellular calcium enter de oocyte or by releasing intracellular calcium reserves. The principal agents used for this purpose are ethanol, electrical impulses, ions and strontium chlorate. The principle concern that arises when using these agents is that they are incapable of generating multiple oscillations and they produce a variation in the amplitude and duration of higher intracellular calcium concentration pulses. In response, there is incomplete degradation of MPF which results in condensation or fragmentation of the transplanted chro-

matin. In contrast to a single stimulus, repetitive calcium spikes induced by artificial means lead to a prompt and stable degradation of MPF in various species [12, 13, 59, 82], indicating that calcium oscillations are required for an effective activation. Furthermore, the calcium-releasing agent introduced by the fertilizing sperm becomes associated with the nucleus up to the 2-cell stage and remains able to induce calcium oscillations, meiotic completion, and pronuclear formation upon transfer back to non-fertilized oocytes [42]. Several alternatives have been studied trying to obtain higher efficiency rates in activation. The use of old oocytes that have a lower capacity to synthesize the enzymes responsible for stabilizing MPF and can therefore be activated with only one intracellular calcium oscillation is one of them. Another option has been the use of protein synthesis inhibitors or enzyme synthesis inhibitors. Some studies with cattle and pork indicate that the use of strontium chlorate in the activation protocol raises the number of embryos that develop to blastocyst stage, when produced by nuclear transfer. On the other hand, comparative studies in cattle and mouse have so far not found any significant differences in cloning efficiency between different oocyte-activating agents [24, 41]. Recently, a combined electric and chemical activation procedure [50] combined with embryo culture in PZM3 resulted in an average of 80–85% blastocyst rates (G. Vajta, unpublished data). This method was also applied for activation of nuclear transfer embryos reconstructed with a zona-free procedure, handmade cloning [43], and contributed significantly to the high overall efficiency of the procedure.

In Vitro Culture of Cloned Embryos

Cloned mammalian embryos are cultured in defined media for various periods of time, usually until reaching the blastocyst stage [57]. Refined in vitro culture systems, where components change in accordance to the need of the embryo [25], allow achieving development rates comparable to IVF embryos. Sadly, this is not accompanied by high in vivo post-implantation survival rates. About three times more IVF than nuclear transfer embryos develop into viable offspring [55].

Embryo Transfer

Although 1 - 4 cloned embryos have been transferred to surrogate mothers, the trend is now to perform single transfers. Since survival rates are comparable, this approach results in a 50-75% cost reduction in producing cloned embryos. The number of embryos transferred to each recipient has also been known to influence the maintenance of pregnancy. Some reports say that transferring twin embryos improves the implantation rate and may result in the birth of twins. Yet, for the bovine species the birth of twins is accompanied by the risk of them being of opposite sex, which may result in the female being sterile by a condition known as “free-martin”. Currently, the norm is for single embryo transfers.

Monitoring

A failure of the placenta to develop and function correctly is a common feature among cattle clones [30], and could possibly due to an inappropriate transition from yolk sac to allantoic nutrition [70]. Others have observed that the growth of the allantois is severely retarded, or even nonexistent, as characterized by lack of, or reduced, vascularization during early gestation, leading to failure of normal placentome development [74]. Although initial day 50 pregnancy rates in cattle following the transfer of single nuclear transfer embryos can be as high as 65%, and similar to both in vitro fertilized embryos and following AI [48], from then on there is a continuing loss of pregnancies. Wells et al. (2004), said only 13% of cloned embryos transferred result in calves delivered at full term. Fetal losses in the bovine species at later stages of gestation are a consequence of placental dysfunction, leading to hydroallantois and the presence of fewer and enlarged placentomes, enlarged umbilical vessels, and edematous placental membranes [93, 45]. The magnitude of this pregnancy failure is in stark contrast to the 0-5% loss post-day 50 with AI or natural mating [19]. Scientists are trying to discover molecular markers that may aid in identifying abnormal placental or fetal development at early stages to diminish the ethical consequences that are associated with the technology.

Recipients pregnant with clones generally show poor preparation for parturition and prolonged gestation, with an increased risk for dystocia from heavier birth weight offspring, often prompting elective caesarean section [93]. Respiratory distress syndromes have been cited frequently in cloned calves [31, 27] and lambs [92], which may indicate poor adrenal gland development and function, low fetal cortisol levels, and, hence, insufficient lung surfactant [71]. However, corticosteroid therapy to induce parturition one week before expected full term has successfully aided fetal maturation, (assisted) vaginal delivery and improved the maternal response towards rearing offspring [89]. Despite having better and faster veterinary

care, cloned calves exhibit reduced survival at delivery and up to weaning when compared to normal calves. Wells et al. (2004), found that around 80% of cloned calves delivered at term are alive after 24 hours, with an additional 15% of calves dying before weaning. Common mortality factors are attributed to dystocia, abnormalities of cardiovascular, musculoskeletal and neurological systems, as well as susceptibility to gastroenteritis, umbilical and respiratory infection and digestive disorders [91, 31, 62]. Cloned embryos and offspring also often show many abnormalities, including circulatory distress, placenta edema, hydrallantois, and chronic pulmonary hypertension [51]. Cloned animals that survive to term frequently suffer from pathological fetal overgrowth, a condition referred to as "large offspring sindrome" (LOS) which is believed to be caused by placental dysfunction. This abnormal development and the low efficiency to which it leads, is mainly due to incomplete reprogramming and abnormal gene expression specially in the genes known to be imprinted.

Epigenetic modification of the genome ensures proper gene activation during development and involves genomic methylation changes, the assembly of histones and histone variants into nucleosomes, and remodeling of other chromatin-associated proteins [63]. The epigenetic structure of the somatic cell nucleus presents marked differences when compared to a mature gamete's nucleus. It is amazing that the oocyte has the ability to reverse epigenetic modifications, imposed on the genome during differentiation, and return to a totipotent state. The initial molecular events that accomplish reprogramming in the mammalian oocyte are still poorly understood, although some observations suggest, that the oocyte cytoplasm is predominantly in control of the initial transcriptional activity of the donor nucleus.
In order to complete development, clones must reactivate genes that are normally expressed during embryogenesis, but remain silent in somatic donor cells. During gametogenesis in normal development, a complex process of epigenetic remodeling assures that the genome of the two gametes, when combined at fertilization, can faithfully activate early embryonic gene expression [63]. In a cloned embryo, reprogramming has to occur in a cellular context radically different from gametogenesis and within the short interval between transfer of the donor nucleus into the egg and the time when zygotic transcription becomes necessary for further development [63].

All the abnormalities that have been discussed, lead to the development of what is commonly known as cloned phenotypes. Possible explanations for the abnormal phenotypes of clones include reprogramming errors, epigenetic damage incurred during in vitro cultivation of embryos before their transfer into the uterus, and undefined parameters of the nuclear transfer procedure itself that could somehow affect development of the clone [63].
Because similar phenotypes have been observed in human patients and in mice as a consequence of both naturally occurring and targeted mutagenesis of imprinted genes, these apparent similarities suggested that aberrant expression of imprinted genes might cause some of the abnormalities seen in clones [34]. The cause of these abnormal clone phenotypes could be due to preexisting epigenetic errors in the donor nucleus or faulty epigenetic reprogramming. Errors in the donor nucleus would be expected to increase with the age of the donor animal and/or the length of in vitro cultivation of the donor cells, whereas faulty reprogramming may depend on the cell type of the donor nucleus [63]. The considerations discussed, portray the possibility that apparently healthy cloned animals may suffer subtle gene expression abnormalities that may not be severe enough to cause lethality or an obvious postnatal phenotype.

Applications

There is still a long way to go before this technology is widely accepted for commercial use on livestock. Important issues concerning animal welfare and health status of cloned livestock and their progeny need to be addressed and made public in order to achieve a general acceptance by farmers, consumers and industries. Advances are however in course to define regulatory approval on the safety of food products deriving from clones and their offspring. Since 2001, when it became apparent that commercial animal cloning could be used to improve the quality of herds, the FDA (U.S. Food and Drug Administration) conducted several intensive evaluations that examined the safety of food products from cloned animals and the potential risk they could pose for human health. After several risk assessment reports, on January 2008, FDA scientist concluded that the meat and milk produced by bovine, porcine and caprine clones or the offspring of cloned animals are as safe as the food produced by sexually reproduced animals.
Valuable Genotypes

Somatic cell cloning can make an important contribution to animal production by enabling to raise identical copies of animals with the best production rates in a herd. This would allow a rapid increase in production after only one generation. Cloning could enable rapid dissemination of superior genotypes from nucleus breeding herds, directly to commercial farmers [57]. Genotypes could be matched to specific product characteristics, environmental conditions or disease resistance. The technology could bring tremendous benefits if used to multiply superior F1 crossbred animals, or composite breeds, to maximize the benefits of heterosis and preventing segregation, in the F2 generation, of favorable allele combinations. Production of cloned animals with superior genetics for breeding, like progeny-tested bulls and sires, would allow the dissemination of genetic gain. The beef industry could benefit greatly from this use by multiplying superior genetics through natural mating instead of AI programs that are inconvenient for extensive farming systems and have high cost of implementation. The dairy industry could produce extra semen from top sire clones in order to meet the unmet international demand of insemination doses. To even more rapidly disseminate genetic gain, and reduce genetic lag by at least two generations, it will be advantageous to clone from embryonic blastomeres following marker assisted genetic selection to identify superior embryos, rather than using somatic cells from adult animals [94].

Preservation

Cloning can be used to help preserve indigenous or traditional breeds of livestock that have production traits and adaptability to local environments that should not be lost from the local gene pool [93]. The extinction of these breeds would mean a significant loss of biodiversity that would limit future opportunities of studying and using traits that are not appreciated today. Even more important would be cryopreservation of somatic cells from rare breeds for future cloning of deceased animals and reintroduction of their genetic backgrounds into the live breeding population [91]. Furthermore, it would be easier to cryobank, using liquid nitrogen, somatic cells as donors for future nuclear transfer than it is preserving embryos and gametes. The possibility of insurance for genetically elite animals would open, by cryopreservation of somatic cells in case of disease or accident related deaths.

Transgenic Animals

The possibility of cloning animals from cells that have been genetically modified is a major application for the nuclear transfer technique, although it is only one of several available methods for transgenic procedures. The reasons for producing transgenic animals are not simple or clear, but it would mean the possibility of deciphering the genetic code and by doing so obtaining large amounts of new information that could open up new research areas. It would give way to the construction of models for studying genetic illness and genetic control over physiological systems. Just as interesting would be producing new animal products and improving animal production traits.

Transgenic mammals were first produced through the microinjection of gene constructs into the pronuclei of fertilized mouse zygotes [7]. However, pronuclear microinjection is only efficient in species with clear cytoplasm that enables the visualization of the pronuclei. Since most mammalian species contain large lipid citoplasmatic vesicles, the method did not achieve satisfying results. The solution was to use transfected cells as nuclear transfer donors to generate transgenic animals. This technique was first applied in sheep and cattle [66, 11] but, recently, has also been used to produce transgenic mice [64] and goats [38]. Fibroblasts are obtained from fetuses and are used to produce a primary cell line, which, once established and checked for chromosomal stability, is transfected by common cell transfection techniques [71]. A selection and reporter gene construct is usually added to the transgene of interest to enable the isolation of suitable cell clones for nuclear transfer [71], which will be those clones that have integrated the transgene and correctly express the reporter gene. The green fluorescent protein (GFP) reporter gene is a suitable marker for transgene integration and expression, because the screening uses blue wavelength exposure, which does not affect further development [71].

The objective of the transgenic technique is to produce animals that posses a stable fragment of exogenous DNA in their germinative linage. These individuals will serve as founders of herds because they will generate progeny that carries one or more of the desirable genes [28]. It is still recommended to use assisted sexual reproduction to further multiply animals without the potential epigenetic aberrations of clones [21].

Practical applications of transgenic animal production include improving composition and overall production of milk, growth rates, increasing feed transformation rates, resistance to diseases, reproductive performance

and biomedicine among others [96]. The combination of gene transfer in cultured somatic cells and somatic cell nuclear transfer techniques provide an attractive alternative to improve transgenic efficiency. To date, more than ten recombinant proteins have been produced in the milk of either goats, sheeps, rabbits or pigs [86]. Furthermore, several functional heterologous proteins, including lysostaphin [87], bovine casein [8] and human lactoferrin [81], have been produced via cattle mammary bioreactors [104]. There has been success in the prevention of mastitis caused by Staphylococcus aureus infection in dairy cattle, after the introduction of a biologically active form of lysostaphin [87], and resistance to ectoparasites may be possible with the production of chitinase in the skin to kill larvae [88].

Conclusiones

Somatic cell cloning is now a reality, with technical and practical uses that need to be studied to greater depth, seeing as they have the ability to revolutionize animal production and research as we know it. Due to the potential advantages for pharmaceutical companies, farmers, and research agencies, mammalian cloning has become a common technology towards the end of this decade and will do so to a greater extent in the years to come. The advances accomplished in the field of cloning and transgenic techniques provides the basis for an exciting future in which large-scale production of nutrients and pharmaceutical proteins using the livestock mammary bioreactor, will be possible and hopefully common. As science discovers new and better applications for these biotechnologies it will be possible to develop models that contribute in the fight against world hunger and health issues affecting the human race. Specially when considering that studies suggest that the composition of milk and meat products and the general health of cloned animals are similar to those produced by gamete mating. This is a window into the future.

On the other hand, the nuclear transfer technique has changed very little in the last ten years, in spite the increasing number of clones that have been and are being produced. Now is the time to strive for the better understanding of the mechanisms responsible for somatic reprogramming in order to improve the efficiency of the procedure. At the same time, it is necessary that veterinary clinicians and theriogenologists focus on finding the source of medical problems associated with cloned animals and develop more effective treatments to reduce neonatal morbidity and mortality levels, as well as pregnancy losses. Only then will it be possible to think of mass scale cloning for industrial purposes that may contribute to ease some of the burdens that plague humanity.

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