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Review

Animal cloning by nuclear transfer: state-of-the-art and future perspectives*

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Model organisms are essential to study the genetic basis of human diseases. Transgenic mammalian models, especially genetic knock-out mice have catalysed the progress in this area. To continue the advancement, further sophisticated and refined models are crucially needed to study the genetic basis and manifestations of numerous human diseases. Coinciding with the start of the new era of post-genomic research, new tools for establishment of transgenesis, such as nuclear transfer and gene targeting in somatic cells, have become available, offering a unique opportunity for the generation of transgenic animal models. The new technology provides important tools for comparative functional genomics to promote the interpretation and increase the practical value of the data generated in numerous mouse models. This paper discusses the state-of-the-art of the nuclear replacement technology and presents future perspectives.

Keywords: cloning, transgenic, animal models, epigenetics

Transgenic animal models have played and are anticipated to continue to play an important role in our pursuit of knowledge of the genetic basis of human disease. There is a need for animal models instead of cell culture because of the complexity of the biological processes that form the basis of most diseases. To-date, most of the available information has been generated in the mouse. The availability of stable embryonic stem cell lines enables gene targeting technology in the mouse. However, the lack of such cell lines in most mammals has so far prevented targeted genetic modifications. Although murine models have been of great use, the potential of the mouse model is limited because the anatomy and physiology of the mouse is not always fully adequate to study many of the diseases affecting the human population. Many species have metabolism and organs with characteristics much closer to that of humans than is the case in mouse (such as lipoprotein metabolism and the development of atherosclerosis in rabbits, skin structure and organ capacity and size for transplantation in pig). However, the lack of stable stem cell lines in animal species other than the mouse has blocked the way for the usage of refined genetic tools for specific targeted genetic models. Furthermore, the difficulties and expenses of the conventional method for generating transgenic models has hindered the progress. The nuclear transfer technology has become available for studies in non-murine species and can be exploited to bridge the gap between mouse models and treatment of human diseases by generating transgenic models in other species.

Despite its great potentials, the nuclear transfer technology is still in its infancy. Some of the reasons for the low efficiency and the possibilities to overcome the present limitations are described below.

STATE OF NUCLEAR TRANSFER

Since the first mammals to be 'cloned' from cultured differentiated cells (Campbell *et al.*, 1996), and the birth of 'Dolly', the first mammal derived from an adult somatic cell (Wilmut *et al.*, 1997) progress was fast. Milestones included the first transgenic mammal to be produced by nuclear

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transfer from a cell line genetically modified in culture (Schnieke *et al.*, 1997); the first nuclear transfer pigs (Polejaeva *et al.*, 2000; Onishi *et al.*, 2000) and the first gene-targeted non-murine mammals (McCreath *et al.*, 2000, first "knock-in" lambs; Denning *et al.*, 2001a, first "knock-out" lamb; Phelps *et al.*, 2003, first knock-out pig). Nuclear replacement efficiency varies among species. In cattle the reported percentage of born progeny increased from the initial 1% to around 20%. This considerable progress is due, besides the improved nuclear replacement methods, to better embryo culture systems. The mouse nuclear transfer technology is relatively well established (Wakayama *et al.*, 1999; Zhou *et al.*, 2001). In rabbit, development of the technology recently resulted in the first somatic-cell nuclear transfer rabbit (Chesne *et al.*, 2002). The technology has also resulted in live birth in other mammals, including rat (Zhou *et al.*, 2003), goat (Baguisi *et al.*, 1999), horse (Galli *et al.*, 2003), mule (Woods *et al.*, 2003), cat (Shin *et al.*, 2002), banteng, gaur (Lanza *et al.*, 2000), African Wildcat (Gomez *et al.*, 2004) and dog (Lee *et al.*, 2005). In human, nuclear transfer embryos have been created, and embryonic stem cell lines established from them for potential cell therapy ("therapeutic cloning", Hwang *et al.*, 2004; 2005).

These successes do, however, mask several confounding issues, including the limited lifespan of primary somatic cells in culture as well as the low efficiency of gene targeting in these cells (Denning *et al.*, 2001b), which effectively limits the target genes to those that are expressed in the donor cell. Complex strategies need to be developed to overcome such restraints.

CRITICAL ASPECTS OF NUCLEAR TRANSFER

Somatic cell nuclear transfer is characterised by a series of developmental abnormalities, the so called cloning-syndrome. This encompasses higher rates of pregnancy loss, prolonged gestation, higher birth weight, higher rates of peri- and post-natal mortality and specific adult phenotypes (Hill *et al.*, 1999; De Sousa *et al.*, 2001). Only about two-thirds of clones delivered at term survive at least until weaning. It has been claimed that at least these long-term survivors can be physiologically normal and apparently healthy, displaying normal behaviour, growth rates, reproduction and productivity. Equally, there are reports of various abnormal cloning-associated phenotypes, including higher annual mortality rates in cattle, reduced maximal lifespan, and obesity in mice (Eggan *et al.*, 2001; Tamashiro *et al.*, 2002), and compromised immune function in both species (Renard *et al.*, 2002). The incidence of these anomalies varies according to the species, genotype, donor cell status, or specific aspects of the nuclear transfer and culture protocols used and it is not clear which of them could be eliminated by technical improvements. Despite the present inefficiencies, mouse nuclear replacement experiments have proven that this method is already capable of improving the efficiency of transgenic production compared to traditional methods (Wakayama *et al.*, 2001).

NUCLEAR REPROGRAMMING AND EPIGENETICS

The reasons behind the frequent deformations and high mortality among nuclear replacement foetuses and progeny are not well understood, but genetic reprogramming problems are likely to be involved. In natural reproduction, relatively low levels of DNA methylation exist in the male and female gametes, which are further demethylated during early embryo development. With nuclear transplantation, the somatic nucleus carries the specific epigenetic modifications of its tissue type, which must be erased during nuclear reprogramming. Therefore, the levels of epigenetic modification existing in donor cells may affect their reprogrammability following nuclear transfer. A discrepancy in the reprogrammability has been observed in different cell types, which results in altered *in vitro* and *in vivo* development of cloned embryos (Kato *et al.*, 2000; Rideout *et al.*, 2000).

Nuclear transfer embryos are known for problems of implantation, abnormalities in placental development and losses during pregnancy. These aberrations are consequences of epigenetic errors, and studies on the frequency of such errors in various genes have revealed that imprinted genes, very important for placental development, are particularly often affected (Rideout *et al.*, 2001). According to recent data, these epigenetic deviations are reset in the second generation after nuclear replacement in conjunction with gametogenesis (Tamashiro *et al.*, 2002). It is, however, an absolute requirement that the first generation of nuclear replacement animals is epigenetically normal enough to complete embryogenesis, foetal development and adaptation to post-natal life.

Mitochondrial inheritance is a specific issue in nuclear replacement and deserves special attention during the development of the technology. However, it is now evident that the offspring generated through nuclear replacement has inconsistent patterns of mtDNA inheritance, often with mixed populations (heteroplasmy) of mtDNA (Takeda *et al.*, 1999; Hiendleder *et al.*, 2003; Steinborn *et al.*, 2000). This heteroplasmy can arise from the introduction of mtDNA accompanying the donor nucleus into the reconstructed embryo and/or the use of recipient oocytes from the ovaries of different females. 'Dolly the lamb' possessed only the recipient egg mtDNA

(Evans *et al.*, 1999). However, in each case, the nuclear donor will remain the same and analysis of nuclear DNA alone will indicate that these offspring are identical.

ADDITIONAL FACTORS AFFECTING NUCLEAR TRANSFER EFFICIENCY

The efficiency of nuclear replacement is also influenced by the donor cells, *in vitro* activation and cultivation methods and technical aspects regarding micromanipulation. The origin of the donor cells affects success rates and survival in culture. For any given somatic cell type, the best source for nuclear transfer is still a matter of debate. The highest cloning efficiencies were achieved with oocytes and donor cells of hybrid mice.

FUTURE PERSPECTIVE

Nuclear transfer is a fast developing technique, far from being optimized. Evolution in the efficiency and applicability in new species is expected. However, it is not clear how much of an improvement can be achieved by simply optimizing the present protocols. Revolutionary changes in our understanding of the reprogramming of the nuclear material, organization of chromatin and epigenetic changes are necessary to allow proper control of the procedures and to avoid epigenetic aberrations in the progeny. Clones of the same donor provide unique experimental material since they are genetically identical yet may be epigenetically different regarding imprinted and X-linked genes. Therefore, the nuclear transfer animal model provides insight into epigenetic regulation that cannot be studied in natural reproduction.

Several new technologies and molecular biological events are studied preferentially in mice, but the practical production of transgenic animals in this species is not likely to be dominated by the nuclear transfer procedures in the near future, as the existing microinjection and embryonic stem cell technology-based methods work efficiently. However, comparison of embryonic stem- and somatic-cell nuclear transfer in mouse will allow a true validation of the nuclear transfer technology for standardized transgenic animal production.

Rat nuclear transfer is one of the biggest technological challenges, partially due to the high sensitivity of rat oocytes to spontaneous parthenogenetic activation. Recently, rat nuclear transfer succeeded as well, using chemicals that block pre-activation before nuclear transfer (Zhou *et al.*, 2003). Use of transgenic rats in the pharmaceutical testing of new drugs might be one of the main activities in the future, and nuclear transfer might represent a viable method to produce stable knock-out and knock-in animals in this important species.

Rabbit nuclear transfer has been successful in producing progeny with fresh cumulus cells, but not with cultured cells. Several transgenic human disease models could be developed in rabbit which are not available in mouse, due to anatomical and physiological differences (e.g., cystic fibrosis, atherosclerosis). Furthermore, placenta structure of the rabbit resembles that of the human, providing a chance for model studies on human reproduction.

Basic research in nuclear reprogramming will continue, especially due to connections to the embryonic stem cell research field. Practical application of nuclear transfer in the near future is expected in the agriculture for enhanced food production and propagation of superior breeding stock; in medicine by using animals as novel disease models, bioreactors and organ donors produced by the combination of transgenesis/homologous recombination in somatic cell lines and nuclear transfer; and in therapeutic cloning of human embryos. Methods with much improved efficiency would provide a good tool for endangered species preservation efforts, as well. In the case of industrial utilization the importance of avoiding inbreeding with advanced breeding management will increase.

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