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▶ **To cite this version:**

Xuemei Li, Ziyi Li, Alice Jouneau, Qi Zhou, Jean Paul J. P. Renard. Nuclear transfer : progress and quandaries. Reproductive Biology and Endocrinology, 2003, 1 (84), 6 p. 10.1186/1477-7827-1-84 . hal-02677996

HAL Id: hal-02677996

https://hal.inrae.fr/hal-02677996

Submitted on 31 May 2020

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Reproductive Biology and Endocrinology

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Review

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Nuclear transfer: Progress and quandaries

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Published: 07 November 2003

Reproductive Biology and Endocrinology 2003, **1**:84

This article is available from: http://www.rbej.com/content/1/1/84

Received: 14 July 2003
Accepted: 07 November 2003

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Abstract

Cloning mammals by nuclear transfer is a powerful technique that is quickly advancing the development of genetically defined animal models. However, the overall efficiency of nuclear transfer is still very low and several hurdles remain before the power of this technique will be fully harnessed. Among these hurdles include an incomplete understanding of biologic processes that control epigenetic reprogramming of the donor genome following nuclear transfer. Incomplete epigenetic reprogramming is considered the major cause of the developmental failure of cloned embryos and is frequently associated with the disregulation of specific genes. At present, little is known about the developmental mechanism of reconstructed embryos. Therefore, screening strategies to design nuclear transfer protocols that will mimic the epigenetic remodeling occurring in normal embryos and identifying molecular parameters that can assess the developmental potential of pre-implantation embryos are becoming increasingly important. A crucial need at present is to understand the molecular events required for efficient reprogramming of donor genomes after nuclear transfer. This knowledge will help to identify the molecular basis of developmental defects seen in cloned embryos and provide methods for circumventing such problems associated with cloning the future application of this technology.

Introduction

Nuclear transfer (NT) is a powerful technique for exploring functional changes in the genome during differentiation. Its application is currently being expanded to generate genetically modified animals. Theoretically, using NT technology can establish a population with genetic characteristics from one genetically modified or genetically specific individual. Although somatic nuclear transfer has been successfully achieved in various species, its efficiency has been very low until recently. The inefficiency lies in many areas, such as in the donor cell types, cell cycle stages, genetic background of donor cells and recipient oocytes, nuclear transfer procedure, and culture environments [1–3]. Because little is known about the developmental mechanisms of reconstructed embryos, it is difficult to optimize the conditions for increasing their efficiency. In this article, we will summarize new advances in NT and the quandaries that have yet to be resolved.

Progress in Nuclear Transfer

The factors involved in the success of NT are very complex. Although many protocols have been modified and utilized in NT processes, some events continue to remain ill-defined. NT in theory attempts to re-establish the "developmental program" of a somatic donor genome to a primordial "ES-cell like" state. 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 [4], mouse [5], cow [6], goat [7], pig [8], rabbit [9], cat [10], mule [11], horse [12], and rat [13]

In all successful NT cloning experiments, the unfertilized cytoplasm of the oocyte was confirmed to be the capable recipient, suggesting that certain factors in unfertilized oocyte are essential for reprogramming the donor genome. Such factors are lost or insufficient following fertilization of the oocyte. The events in the first several cell cycles of cloned embryos, therefore, are critical to the establishment of fully reprogrammed genomes. Gastrulation is a vital stage for cloned embryos. A high rate of abortion during gestation has been observed in cloning experiments performed on different species [5,16,17]. These late miscarriages are also frequently associated with abnormal development of the placenta [18]. Cloned embryos and offspring also often show many abnormalities, including circulatory distress, placenta edema, hydrallantois, and chronic pulmonary hypertension. The surviving offspring also have large placentas and increased birth weights, and they suffer a high incidence of death [14,15].

The low efficiency and abnormal development of cloned animals are mainly due to incomplete reprogramming and abnormal gene expression. The expression of several important genes has been assessed in cloned embryos [19,20]. In a study in which global gene expression was analyzed by microarray in NT embryos derived from stem cells and somatic cells, 4% of more than 10,000 genes differed in expression from the controls [21].

Quandaries in Nuclear Transfer

Although some achievements have been made in the field of nuclear transfer, many quandaries still persist. One example is the aberrant reprogramming observed in physically normal cloned adults.

Aberrant Reprogramming and Physically Normal Cloned Adults

Considering the frequency of abnormal gene expression, it could be considered surprising that physically, functionally, and histologically normal cloned adults have been produced in several species. When a fertile cloned animal is delivered, the donor cells should be reprogrammed into a state compatible with embryonic development. However, most cloned embryos have been observed to fail to develop to term, and some of the surviving cloned animals have shown abnormalities. The major cause may reside in faulty or incomplete epigenetic reprogramming of the donor nucleus, which affects the gene expression needed for every developmental stage of cloned embryos and offspring. Most cloned embryos lose their developmental abilities during pre-implantation and gastrulation. Moreover, the surviving adults often show abnormalities.

To better understand the issues controlling incomplete epigenetic reprogramming, we have compared the long-term viability of mice derived from ES nuclei and somatic nuclei. Reconstructed embryos were transferred into foster mothers, and caesarean sections were performed at day 19 of gestation. The combined weight of placentas for all cloned pups obtained was approximately double the weight of the control. From the standpoint of live birth cloned pups, more than half of the pups suffered from respiratory failure and general weakness and died only a few hours after delivery. The weight curves of NT mice that did survive were followed for 12 to 19 months and were similar to those of the controls. This study also resulted in two infertile cloned mice out of 9, both of which could mate and give vaginal plugs normally; however, histology of the testicle of one of them showed a reduced number of germinal cells with no spermatozoa detectable in the epididymis, and surprisingly, spermatozoa were found in the epididymis of the other male, although it was proven not to be able to produce fertilised zygotes after natural mating. Some mice died after one year, suffering from multiple necrotic wounds. In order to obtain physical parameters, we sacrificed some cloned mice at different ages (1, 8, and 19 months old) and compared them to control animals of the same ages. Blood count and formula were determined, and several organs (the lung, liver, spleen, testis, and kidney) were processed for histology. No marked differences could be detected between normal and cloned animals. Of the seven NT animals, only two were overweight, compared to one out of five for the controls. Our data, together with the fact that one of our first NT mice obtained from somatic nuclei (cumulus) was still physiologically normal before mercy killing at two and half years, provide evidence that nuclear transfer, despite multiple disorders, can result in physiologically normal, fertile animals.

Although some apparently normal cloned mice were produced, an aberrant expression pattern in NT embryos was observed even in these mice. This pattern concerned genes thought to be involved in stress adaptation, trophoblastic function, and DNA methylation during pre-implantation development. It has been shown that the culture of pre-implantation embryos affects the regulation of various imprinted and nonimprinted genes, leading to aberrant fetal growth and development [22–24].

No significant correlation between the anomalous fetal growth of cloned mice and abnormal expression of any single gene was seen [25]. The accumulated actions of abnormal gene expression at multiple loci ultimately

Table 1: The Postimplantation Development of Mouse-Cloned Embryos Derived from Different Cell Cycle Stages

Donor cell cycle	No. of transferred	No. of implanted (%)	No. of pups			No. of survival after 1 week	
			Total	% from implanted	% from transferred	Total	% of born pups
G2	276	63 (23)	5	10	2.2	2	40
G1	189	30(16)	3	10	1.6	1	33
M phase	1062	265(25)	21	7	2	8	38

resulted in embryonic or postnatal abnormalities. The high incidence of embryonic loss after implantation and postnatal death in clones suggests a need for characterizing molecular parameters that can be used to assess the developmental potential of pre-implantation embryos. Researchers are currently experimenting with different methods of identifying gene expression in nuclear transfer.

A cohort of such identified genes will provide a useful tool when analyzing the developmental potential of pre-implantation embryos. Another valued marker when assessing embryonic developmental potential is genomic methylation. Shi reported that aberrant methylation patterns at the two-cell stage zygote are an indicator of early developmental failure [26]. These authors have used an antibody to 5-methylcytosine to examine the immunostaining patterns of methylated genomic sites in two-cell zygotes developed from superovulated females, nonsuperovulated matings, and *in vitro* fertilization. A major conclusion of their work is that a methylcytosine staining pattern has been shown to be a valuable indicator of early developmental methylation reprogramming of the two parental genomes in normal or *in vitro* fertilized zygotes. The authors indicate that this immunostaining approach promises to be potentially useful for determining the safety and efficiency of technologies that assist with reproduction. To best explain aberrant reprogramming and the acquisition of apparently normal adult animals, there is an increasing need to explore how NT is affected by varying genetic backgrounds, the nuclear transfer procedure, and the synchronization of donors and recipients.

Cell Cycle Coordination and NT Efficiency

Cell cycle synchronization has traditionally been thought the best way to improve the efficiencies of nuclear transfer. The benefit of using early-stage donor nuclei was confirmed by the enhanced rate of development of manipulated embryos to blastocysts with donor blastomeres in the early cell cycle stage (G1). Bypassing the S phase was also considered important for effective nuclear transfer [27,28].

After the delivery of the first cloned adult mammal, Dolly (which was produced by inducing donor nuclei into the quiescent state [4]), many living offspring were produced using quiescent, cultured donor cells [7,29–31]. It is generally believed that a diploid, G0/G1 stage of the cell cycle is required to initiate reprogramming following transfer of the donor nucleus into an inactivated, oocyte cytoplasm. This stage is also thought to ensure that the diploid of the cloned embryo is normal. Other groups have used cycling cells in presumptive G1 stage and have also obtained offspring [32]. As the majority of cumulus cells are presumed to be in the G0/G1 stage, they have also been used for donor cells [5].

We have found that the cell cycle stage of the donor cells could significantly interfere with *in vitro* development of stem cell generated NT embryos [33]. However, the implantation rate at day 7 is quite similar between the three types of nuclei (G2, 23%; G1, 16%; and M-phase, 25%). The pup delivery rates are also similar between metaphase and interphase groups at day 19 (M-phase, 2.0% vs. I-phase G1, 1.6% and G2, 1.9%). The survival rate of the cloned pups after one week is also similar between these two groups (M-phase, 38% vs. I-phase G1, 33% and G2, 40%). However, our results show that up to 85.1% of the cloned embryos develop to blastocysts when metaphase nuclei are injected, whereas this rate drops to about 20% when interphase nuclei are used (G1 and G2) (Table 1).

What does cell cycle synchronization alter? Evidence suggests that cell cycle synchronization can only change the rate of blastocyst formation. When we examined the chromatin remodeling of the injected nucleus during activation, we found that metaphase donor nuclei reformed a metaphase plate rapidly after transferring. Although 20% of the spindles were abnormal, with disordered chromosomal arrangement, 93.3% could form one pseudo pronucleus (PN) and one polar body (PB) 6 hrs after activation. Interphase nuclei underwent premature chromatin condensation (PCC), after which only 50% of the G1 formed 2 PN and 63% of the G2 formed one PN and one PB. In 20% of the cloned embryos derived from

interphase donor nuclei, fragment chromatin and condensed chromatin block were found.

MII cytoplasm is known to induce donor chromatin remodeling, a process that greatly depends upon the donor cell cycle stage. Our research shows that cell cycle synchronization changes the pattern of chromatin remodeling. By avoiding PCC, which may induce chromosomal abnormalities, metaphase donor cells are able to achieve a higher *in vitro* development rate. However, although preimplantation development improved significantly in our research, post-implantation and full-term development were similar in every cell cycle stage analyzed. These data indicate that restoration of the nuclear totipotency depends more on the nature of the donor nucleus than its initial cell cycle stage.

Embryonic and Adult Nuclei – Which Is Easier to Reprogram?

Before Dolly was born, only embryonic nuclei could be transferred and could reach full-term development; now, somatic nuclear transfer is a routine procedure. Various differentiated cell types have been used as sources of nuclei for cloning domestic and laboratory animals. Almost all cell types tested have resulted in live offspring, although with great differences in efficiency. Usually, the development of cloned embryos receiving a well-differentiated donor nuclei is less successful than for those transferred with low-differentiated donors. The survival until birth and adulthood of blastocysts that are derived from ES-cell, donor nuclei is much higher than for clones from somatic donor nuclei [34,35]. We have also found that ES cells seem to provide 20 times better development than cumulus cells [33,36]. Many developmentally important genes have also been detected in cloned embryos. Bortivin et al. [37] analyzed expression of *Oct4* and 10 *Oct4*-related genes in individual, cumulus, cell-derived, cloned blastocysts. They found that only 62% correctly expressed all tested genes. In contrast to this incomplete reactivation of *Oct4*-related genes in somatic clones, ES cell-derived, cloned blastocysts and normal control embryos expressed these genes normally. These authors postulated that clones derived from differentiated cell nuclei might fail to establish a population of truly pluripotent embryonic cells due to faulty reactivation of key embryonic genes.

It can be difficult to explain the development of cloned embryos solely by their differentiated states. Therefore, we tested ES cells and fibroblast cells with the same genotype (129/SvPas), synchronizing the donor nuclei from ES cells and somatic cells in metaphase. Pre-implantation and postimplantation development was checked at the blastocyst stage and at day 7.5 of pregnancy. We found that although there was an approximate 10-fold difference at the blastocyst stage between ES cells and fibroblast cells, the implantation rate between the two groups was only an approximate two-fold difference. Surprisingly, the fetus rate (implantation with embryos from blastocyst transferring) at day 7.5 was three-fold lower (Table 2). The level of methylation is likely implicated in this result. ES cells have a high methylation activity compared to that of somatic cells [38]. To what extent the extensive demethylation of chromatin during pre-implantation development interferes with the remethylation of the genome during postimplantation stages remains to be determined.

Table 2: Developmental Abilities of Embryos Reconstructed from ES cells and Somatic Cells with the Same Genetic Background (129/Svpas)

	Development of cloned embryos at different stages (%)	
	ES cell	Fibroblast cell
Blastocyst formation	50.0	6.0
Implantation	16.0	8.7
Fetus /embryo transferred	3.4	1.5
Fetus / implantation	29.0	17.1
Fetus / blastocyst	7.2	22.6

Similar epigenetic phenomena have also frequently been observed between subcultures (batches) derived from the same biopsy (thus from the same genotype and same differentiated state) for the bovine species [3] and for clonal cell lines of fibroblasts derived from the same pig fetus [39]. Daniels et al. [19,40] studied the expression of FGF4, FGFr2, and IL6 in the bovine nuclear transfer embryos reconstructed from granulosa and fetal epithelial cells. The authors detected aberrant expression of all three genes in bovine, granulosa, cell-derived, nuclear transfer embryos, but only the expression of FGF4 was observed to be aberrant in the fetal epithelial cell. Their results demonstrate the effects that different donor cell lines and different nuclear transfer procedures may have on the expression of developmentally important genes in nuclear transfer embryos.

In addition to epigenetic change, the nuclear transfer procedure and *in vitro* cultures of the reconstructed embryos also contribute to aberrant gene expression. Cox G.F. et al. [41] suggested that intracytoplasmic sperm injections might increase the risk of imprinting defects. Wrenzycki et al. [20] detected eight specific mRNAs in single blastocysts employing a semiquantitative, reverse transcription-polymerase chain-reaction assay using different nuclear transfer procedures. Their results showed that depending on the steps of the cloning procedure, nuclear transfer-derived embryos might display marked differences from their *in vitro*-produced, *in vivo*-derived counterparts.

Future Prospects

Despite the increasing number of cloned animals produced, the nuclear transfer technique itself has changed little in the last ten years. Modification of the present procedure is required to improve efficiency. As a field, NT cloning must strive to better understand the mechanisms responsible for the currently variable somatic reprogramming to an embryonic or totipotent state. Through a systematic analysis of the molecular events controlling reprogramming of a donor genome will emerge highly efficient methods for NT cloning. Such advances will undoubtedly benefit the field of animal modeling using this technology.

Acknowledgments

We are grateful to Dr. John F. Engelhardt for his suggestions and comments on the manuscript.

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