

Articles

Somatic cell haploidization: an update



Jan Tesarik obtained his MD degree in 1979 and PhD in 1982. He realized the first successful gamete intra-Fallopian transfer (GIFT) and the first childbirths after oocyte fertilization with round spermatids (1995) and with in-vitro cultured spermatids from a man with meiotic maturation arrest (1998). He developed an original technique for nuclear transfer in mature human oocytes (2000). He is author or co-author of >250 scientific publications. At present he is director of MAR&Gen (Molecular Assisted Reproduction and Genetics) in Granada (Spain) and scientific consultant for the Laboratoire d'Eylau (Paris, France) and the European Hospital (Rome, Italy).

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Abstract

Oocyte donation is the only method of treating female sterility caused by complete absence of oocytes, with the loss of genetic motherhood. Genetic fatherhood of males with complete absence of spermatozoa can only be restored by assisted reproduction treatment if sperm precursor cells belonging to the male germline can still be recovered from the testis. Otherwise, sperm donation is the only available solution. Somatic nucleus haploidization after injection into previously enucleated donor oocytes (diploid-to-haploid reduction) might enable the reconstruction of new oocytes carrying the complete nuclear genome of female patients lacking their own oocytes. Such newly formed oocytes could subsequently be fertilized by spermatozoa from the patient's husband. In cases of male infertility with complete absence of the germline, the patient's somatic cell nuclei could be injected into the oocytes without previous enucleation, and somatic nucleus haploidization would occur in the presence of the original female nucleus (triploid-to-diploid reduction), hopefully leading to the formation of a diploid embryo. Both interventions differ substantially from cloning because embryos are formed by syngamy with the male and female genomes originating from the two genetic parents, as in natural fertilization. Ultrastructural remodelling of mouse somatic cell nucleoli can be achieved in enucleated metaphase II mouse oocytes. Haploidization has also been attempted with Sertoli cells and with fibroblasts, both of which are also available in male patients. Experiments are currently under way to assess the regularity of chromatid segregation during somatic nucleus haploidization.

Keywords: non-obstructive azoospermia, oocyte donation, oocyte reconstruction, ovarian failure, somatic nucleus haploidization

Introduction

The cytoplasm of mammalian metaphase II oocytes has many potential capacities that are not shared by metaphase cytoplasm of somatic cells. This has been known since the late 1980s, when it was shown that somatic cell (thymocyte) nuclei injected into metaphase II mouse oocytes skip the DNA synthesis phase and undergo premature condensation (Szollosi *et al.*, 1986). Thus, metaphase II cytoplasm can force somatic cell nuclei to bypass an otherwise obligatory cell cycle checkpoint, which, under normal conditions, prevents the nucleus from metaphase entry before having completed the DNA synthesis phase (Fulka *et al.*, 2000). This capacity of metaphase II ooplasm was exploited in attempts to reconstruct oocytes containing her own genetic material in a woman whose oocytes were not recovered after controlled ovarian

stimulation; this was done by injecting the patient's cumulus cell nuclei into enucleated donor oocytes (Tesarik *et al.*, 2001). The same approach, except that somatic cell nuclei were introduced to entire (non-enucleated) oocytes, was tested in the mouse model for eventual use in cases in which the male germline was lacking, with the somatic nucleus substituting for the male gamete in embryo creation (Lacham Kaplan *et al.*, 2001). Other studies were aimed at the reconstruction of oocytes using an alternative approach based on the introduction of somatic cell nuclei to enucleated immature (germinal vesicle) oocytes (Tsai *et al.*, 2000).

The techniques of assisted reproduction using haploidized somatic cells as substitutes for the female or the male gamete, including the possible future therapeutic potential, biological characteristics of reductional cell division with regard to the

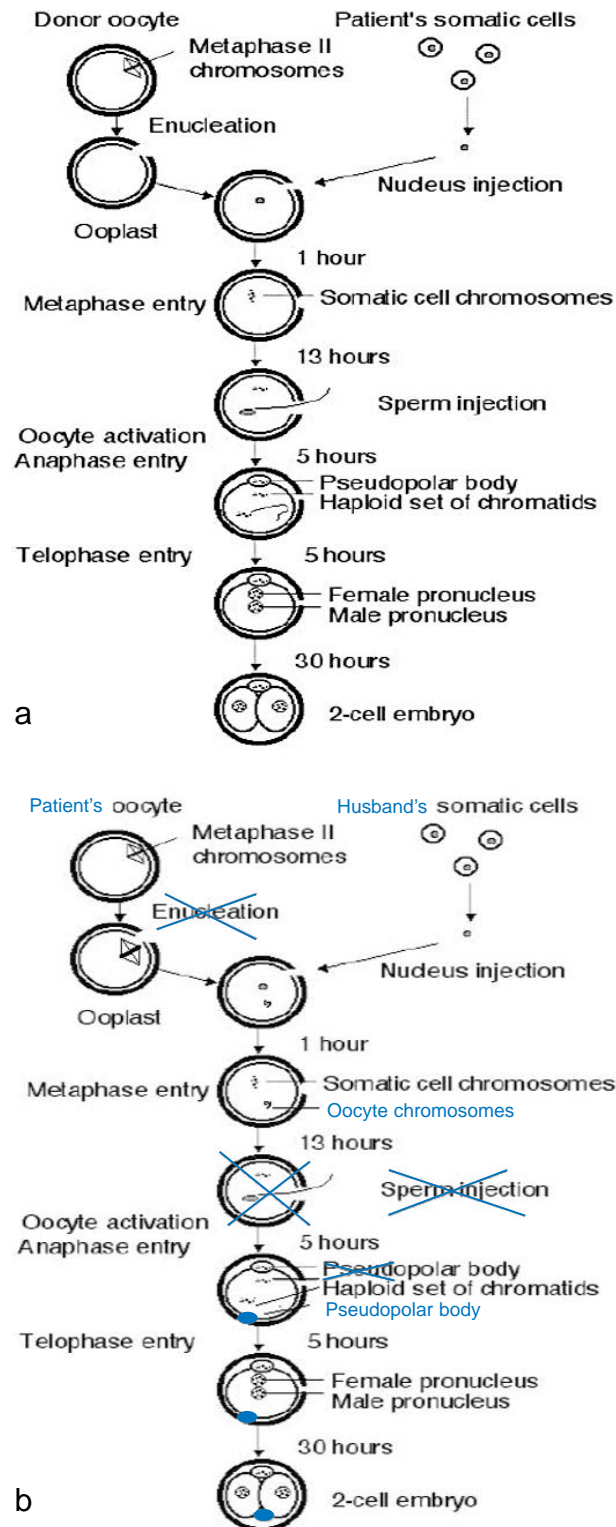


Figure 1. Schematic representation of the similarities and differences between the use of haploidized somatic cells to substitute for the female (a) and the male (b) gamete. Part a represents the design of human oocyte reconstruction from somatic cells and is reproduced from Tesarik *et al.* (2001). Part b shows the modifications with which this design is applicable to fertilization of oocytes, with somatic cells as substitutes for spermatozoa. The differences with respect to part a are highlighted in blue.

classical concept of meiosis, issues related to the function of centrosome and genomic imprinting, and the substantial differences that distinguish these techniques from cloning, have been critically reviewed recently (Tesarik, 2002). The present study focuses on some new biological and technical aspects of somatic cell haploidization and presents an update of experimental data relevant to this exciting new technique.

Experimental design

Substitution for the female gamete (oocyte reconstruction)

Two different schemes for oocyte reconstruction via somatic cell haploidization have been suggested. One is based on the introduction of a somatic cell nucleus in G1 or G0 phase of the cell cycle, originating from a patient lacking her own oocytes, to an enucleated metaphase II oocyte from a healthy donor, taking care to avoid simultaneous oocyte activation. After some time spent in the non-activated state, required for premature chromosome condensation and reprogramming of the somatic nucleus, the oocyte is activated by intracytoplasmic sperm injection (ICSI) using spermatozoa from the patient's husband. This is expected to lead to the extrusion of one haploid set of somatic cell chromosomes to a pseudo-second polar body (Figure 1A). The remaining haploid set of somatic cell chromosomes, together with the haploid set of sperm-derived chromosomes, would thus form the diploid nucleus of the future embryo.

The other approach is based on the introduction of a somatic cell nucleus in S-phase or G2-phase to an enucleated germinal vesicle oocyte. The somatic cell nucleus is then expected to undergo chromosome reduction during two sequential pseudo-meiotic divisions, in a similar way to normal meiosis.

In the former approach, each of the somatic cell chromosomes prematurely driven to metaphase consists of a single chromatid only because no DNA replication has occurred between the nucleus isolation from its mother cell at the G1/G0 phase and the premature metaphase. Thus, there is no mechanical role for recombination and the formation of chiasmata, which during normal meiosis, reinforce the cohesion between homologous chromosomes and thus prevent their premature segregation, which would lead to aneuploidy. On the other hand, the fidelity of the segregation of single-chromatid homologous chromosomes during this artificial haploidization will be critically dependent on their correct alignment on the pseudo-second meiotic spindle.

Substitution for the male gamete (fertilization with somatic cell)

When the somatic cell nucleus originating from a male lacking the germline is used as a substitute for the male gamete, it has to be introduced to an intact metaphase II oocyte that still possesses its genetic material. This inevitably leads to the formation of a triploid zygote, which is diploidized only later, by a mechanism analogous to that described in the previous section (Figure 1B). There is, however, one substantial difference from the use of somatic cells as a substitute for female gametes, since the resulting construct has to be activated by entirely artificial means, without any contribution

from a spermatozoon. The sperm centriole is important, in most mammalian species, for oocyte microtubule organization after fertilization, leading to proper and timely migration and alignment of chromatin. This function will also be lacking if the technique of fertilization with haploidized somatic cells is applied in humans. However, the consequences of sperm centriole deficiency cannot be evaluated in the mouse model because mouse embryos inherit the microtubule organizing centre from the oocyte.

Micromanipulation techniques

The means whereby the somatic cell nucleus is introduced to an intact or enucleated oocyte is very important and will determine the choice of further procedures to ensure the viability of the future embryo. Small nuclei, such as those of cumulus cells or small lymphocytes, can be easily transferred to oocytes by means of microinjection. Under normal conditions, the mechanical manipulation of the oocyte and the influx of calcium-rich external medium during the microinjection procedure do not represent a sufficiently strong stimulus to induce oocyte activation (Tesarik *et al.*, 1994). Hence, the time between the somatic nucleus injection and oocyte activation can be deliberately varied to search for the best setting for optimal chromatin reprogramming.

The introduction of larger nuclei, such as those of Sertoli cells or fibroblasts, by microinjection is more difficult because larger and thus more traumatic microinjection needles and potentially more harmful manipulation techniques have to be used. This may require recourse to a cell–cell fusion technique. However, the most commonly used fusion technique, electrofusion, readily activates metaphase II oocytes of various mammalian species, including humans (Cohen *et al.*, 1998; Tesarik *et al.*, 2000). This represents a serious obstacle because the somatic nucleus needs to spend some time in metaphase II ooplasm in order to undergo premature chromosome condensation and reprogramming. On the other hand, this problem does not occur with methods that use germinal vesicle rather than metaphase II oocytes as vehicles for somatic nucleus haploidization.

In any event, with the use of the piezo system, there are few limitations to the direct injection of cells, even large ones, into the cytoplasm of animal oocytes. Even though there may be some concern about the use of the piezo system for humans, related to the presence of mercury within the system, human oocytes are very versatile compared with oocytes of other mammalian species, and direct injection of large cells using conventional injection methods will probably not be an insurmountable problem.

Cell cycle phase of the nuclear donor cell and recipient oocyte cytoplasm

When the system based on enucleated germinal vesicle oocytes is used for somatic cell nucleus haploidization, the cell cycle phase of the somatic cell should ideally match that of the germinal vesicle oocyte. Germinal vesicle oocytes are in G2-phase of the cell cycle. Accordingly, the somatic cell nuclei should also be synchronized in G2.

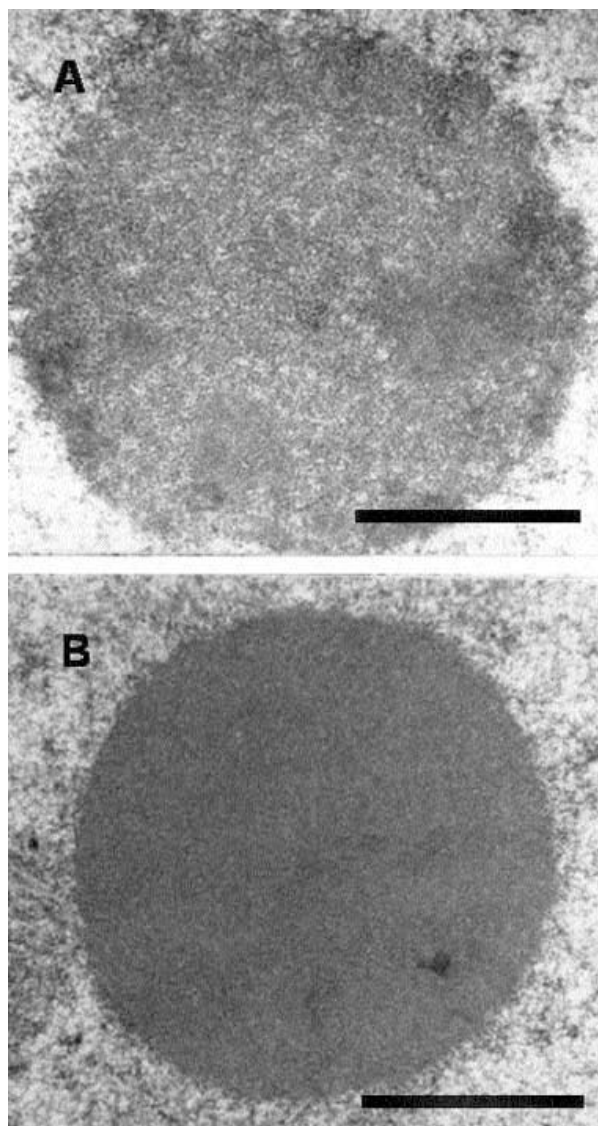


Figure 2. Electron micrographs showing the active nucleolus of a mouse cumulus cell with distinct fibrillar and granular components (a) and a nucleolar precursor body of a pronucleus resulting from remodelling of a cumulus cell nucleus in mouse metaphase II ooplasm (b). Cumulus cells released from pre-ovulatory oocyte–cumulus complexes recovered from superovulated females were injected into ooplasts resulting from enucleation of metaphase II oocytes. The efficiency of enucleation was confirmed by Hoechst staining of the corresponding karyoplasts. After 6 h of incubation, the reconstructed oocytes were activated with 5 $\mu\text{mol/l}$ ionomycin, and they were processed for transmission electron microscopy 12 h later. Previously described methods were used for the recovery, culture and manipulation of cumulus cells and oocytes (Fulka *et al.*, 2002) and for sample processing for electron microscopy (Tesarik and Kopečný, 1989). Bar = 1 μm .

When somatic cell nuclei are to be haploidized after introduction to metaphase II oocytes, the corresponding chromosome configuration cannot be found in somatic cells because the structure of prematurely condensed somatic cell chromosomes is unique to this situation. However, the set of prematurely condensed, single-chromatid somatic cell chromosomes, resulting from the cell cycle checkpoint skip after the insertion of G1/G0 somatic nuclei to metaphase II ooplasm, is comparable to metaphase II chromosomes with regard to overall DNA content. The subsequent segregation, occurring after oocyte activation, will then separate single-chromatid homologous chromosomes and not chromatids as in the second meiosis, although it may lead to the same end result, the formation of a haploid set of single-chromatid chromosomes (Tesarik, 2002). The mechanisms that influence the fidelity of chromosome segregation in this unprecedented reductional division are unlikely to be the same as those controlling normal meiosis, and their nature and efficacy remain to be determined.

Update on experimental data

The feasibility of assisted reproduction with haploidized somatic cells will obviously depend on the solution of multiple questions, most of which, including the oocyte activation mechanism, the interaction of centrosomes originating from different cell types, nuclear reprogramming and genomic imprinting, are also relevant to the current development of mammalian cloning techniques (Tesarik, 2002). However, the crucial problem, on the solution of which the possibilities of further development of technologies based on somatic cell nucleus haploidization will depend, is that of fidelity of homologous chromosome segregation and the extrusion of the proper sets of chromosomes to the pseudo-polar bodies.

The issue of somatic chromosome behaviour in oocyte cytoplasm has been addressed in two animal species, mice and cattle. Pseudo-polar body extrusion was observed in most oocytes that were reconstructed with somatic cell nuclei at the germinal vesicle (Palermo *et al.*, 2002) or metaphase II (Takeuchi T, Rosenwaks Z, Palermo GD, personal communication) stage. Cytogenetic analysis was performed with only a small number of embryos resulting from fertilization of oocytes reconstructed from haploidized somatic cells. Out of 14 embryos resulting from ICSI of mouse oocytes reconstructed with mouse cumulus cells, 12 presented with numerical chromosomal abnormalities and two appeared to be normal. In another study (Heindryckx B, Lierman S, Rybouchkin A, Van der Elst J, Dhont M, personal communication), enucleated and non-enucleated mouse oocytes were used to haploidize cumulus cell nuclei, but the embryonic development was triggered by artificial oocyte activation instead of ICSI. These authors analysed cytogenetically 28 parthenogenetic embryos resulting from oocytes reconstructed after enucleation and 56 embryos from oocytes reconstructed without previous enucleation. The expected chromosome number (20 in the former case and 40 in the latter) was found in six (21%) and 10 (18%) embryos in the two respective groups. In both of these studies, the haploidization strategy based on the injection of somatic cell nuclei to metaphase II ooplasm was used.

In a study using bovine oocytes enucleated in metaphase II, haploidization of adult female fibroblasts was attempted (Ambroggio JD, Beyhan Z, First NL, personal communication). Of 12 oocytes manipulated, reconstruction was successful in two oocytes that were subsequently activated and analysed by Hoechst staining. One of these oocytes appeared to be haploid. However, it remains doubtful how clearly a single chromosome can be identified by Hoechst staining, and further studies using more accurate cytogenetic methods are required to determine the number of chromosomes in embryos resulting from oocytes reconstructed with haploidized somatic cell nuclei.

In another study using bovine oocytes (Nagy ZP, Bourg de Mello MR, Tesarik J, Visentin JA, Abdelmassih R, unpublished work), 52 of 63 enucleated metaphase II oocytes injected with cumulus cells (82%) were reconstructed successfully and injected with bull spermatozoa. Eighteen of the 52 sperm-injected oocytes extruded a pseudo-polar body and 12 of them cleaved. Evaluation of chromosome number in the embryonic cells or polar bodies was not performed in that study.

Recently, a preliminary series of experiments was evaluated in which mouse cumulus cell nuclei were injected into enucleated metaphase II mouse oocytes. After 6 h of incubation, the reconstructed oocytes were activated with ionomycin, incubated for an additional 12 h and examined by transmission electron microscopy (**Figure 2**). In nine of 18 successfully reconstructed oocytes, complete ultrastructural remodelling of cumulus cell nucleoli into pronuclear-type nucleolar precursor bodies was observed (**Figure 2**).

In spite of the preliminary character of these studies and the relatively small numbers of oocytes and embryos analysed, taken together these data strongly suggest that correct segregation of somatic cell chromosomes can be achieved in maturing and mature mammalian oocytes, albeit with a still rather low efficacy. These data also corroborate the findings of a previous study with human oocytes, in which pseudo-second polar bodies extruded by two oocytes reconstructed from human cumulus cells were analysed by FISH for five different chromosomes, and showed the correct number of FISH signals (Tesarik *et al.*, 2001). The question now arises as to whether more balanced chromosome behaviour in reconstructed oocytes can be achieved by modifying technical and biological aspects of the procedure, such as the choice of cell cycle phase of somatic nuclear donor cells and oocytes, the type of the somatic cell used, the method of oocyte activation, culture conditions, etc.

The importance of the oocyte cell cycle phase is suggested by recent observations on the behaviour of somatic cell derived chromosomes after their introduction to mouse oocytes at the germinal vesicle stage (Fulka *et al.*, 2002). It was found that chromosomes originating from somatic (cumulus cell) nuclei only rarely arrange in normally shaped metaphase plates, but rather show chaotic behaviour, arrange in abortive metaphase plates and generally fail to be extruded to a pseudo-first polar body in anaphase of meiosis I (Fulka *et al.*, 2002). These data suggest that the design using germinal vesicle oocytes for somatic nucleus haploidization is particularly prone to segregation errors, possibly because of the lack of

recombination sites and chiasmata on mitotic chromosomes. This could result in the lack of cohesion between homologous chromosomes. The lack of synapsis may also contribute to the picture by compromising the proper chromosome alignment on the meiotic spindle. Interestingly, a similar picture of disorganized meiotic spindles has been observed in metaphase I oocytes from mice carrying meiotic mutation *mei1* which disrupts chromosome synapsis (Libby *et al.*, 2002).

A study evaluating the behaviour of somatic cell chromosomes after their introduction to metaphase II mouse oocytes, as an alternative approach to somatic cell nucleus haploidization, is currently under progress. Preliminary results from this series of experiments show markedly better results in terms of pseudo-polar body formation and chromosome distribution on the meiotic spindle.

Existing data and future perspectives

Fidelity of reductional division

Even though the existing data suggest that assisted reproduction with haploidized somatic cells may become a feasible option in the future, they still are unable to dissipate major concerns about the efficacy and safety of this approach. Fidelity of somatic cell-derived chromosome segregation during the pseudo-meiotic reductional division, the completeness of nuclear reprogramming and the proper function of epigenetic factors determining embryonic and fetal development are the most frequently raised points.

Studies performed in different laboratories and with cells from different mammalian species (see above) suggest that correct segregation of somatic cell chromosomes between the injected ooplast and the pseudo-polar body occurs in some cases, but they also demonstrate the extreme fragility of this process in the current laboratory setting. In fact, most embryos resulting from haploidized somatic cells are apparently aneuploid.

Several approaches can be suggested to cope with this problem. M-phase checkpoint controls, which normally prevent cells with erroneously segregated chromosomes from entering anaphase, are relatively inefficient in female meiosis I (Fulka *et al.*, 2000). Hence, eventual errors generated in the haploidization system using germinal vesicle oocytes are unlikely to be corrected. As to the system using metaphase II oocytes, any existing experimental data relative to the second meiotic division are only marginally relevant because the separation of two homologous, one-chromatid chromosomes originating from a somatic cell is a process substantially different from meiosis II (Tesarik, 2002).

Because important cell cycle molecules are preferentially associated with spindle structures (Kubiak *et al.*, 1993), they are mostly removed from metaphase II oocytes at enucleation. Thus, the control of chromosome segregation during somatic cell nucleus haploidization in metaphase II ooplasm is likely to be different in intact (somatic cell substitution for the male gamete) and enucleated (somatic cell substitution for the female gamete) oocytes. In somatic cells, cell cycle progression from metaphase to anaphase is blocked by the

presence of unattached kinetochores (Amon, 1999) that bind the checkpoint components Mad2, Bub1 and Bub3 and, with the probable participation of Mad1, Mad3 and other proteins, promote the formation of an inactive Cdc20-APC-Mad2 complex (Fulka *et al.*, 2000). When all kinetochores are attached to microtubules, Mad2 and probably some other components dissociate from CDC20-APC which thereafter degrades the anaphase inhibitor Pds1. Some of these proteins may be present in metaphase II ooplasm and others may be associated with the somatic cell nuclei to be haploidized. The efficacy of the hypothetical checkpoint controlling the fidelity of chromosome segregation during haploidization will also probably require a certain degree of cohesion between homologous chromosomes, so as to prevent premature movement of recently spindle-attached kinetochores towards the spindle poles. In fact, a temporary equilibrium between the spindle traction forces and interchromosome cohesion may be required to retain the metaphase configuration for the time necessary for the checkpoint controls to act. This indeed seemed to take place when cumulus cells were used for oocyte reconstruction, because neither pronuclei nor a pseudo-polar body appeared after nucleus injection into metaphase II ooplasts until the construct was activated by subsequent sperm injection (Tesarik *et al.*, 2001). By contrast, the injection of Sertoli cell nuclei under the same conditions usually did not lead to pseudo-second polar body extrusion and, after oocyte activation, the Sertoli cell-derived chromatin became dispersed in multiple pronuclei (unpublished data). These observations suggest that nuclei from different types of somatic cells differ as to the capacity to be haploidized in metaphase II ooplasm, possibly depending on the presence and quantity of cell cycle-controlling or interchromosome cohesion-promoting proteins. The eventual recourse to artificial enrichment of metaphase II ooplasm in some of these proteins by injecting the protein itself or the corresponding mRNA remains a challenge for future research.

Nuclear and epigenetic reprogramming

Unlike the fidelity of chromosome segregation during somatic cell haploidization, which is an issue unique to this particular approach, other major questions relative to haploidization, particularly those of nuclear and epigenetic reprogramming, are common to both haploidization and conventional cloning. Nuclear reprogramming is a vaguely defined term that refers to the capacity of somatic cell nuclei, whose transcriptional activity has been restricted to a cell type-specific pattern, to backtrack to the unrestricted, polyvalent transcriptional pattern.

Nuclear reprogramming is obviously a complex process whose individual events are difficult to trace. However, the efficiency of reprogramming can be estimated indirectly, by examining structural correlates of nuclear activity. For instance, remodelling of somatic cell nuclei to patterns similar to those seen in pronuclei is generally considered an early sign of reprogramming. Interestingly, fertilization of human oocytes reconstructed with cumulus cell nuclei resulted in the formation of zygotes with two structurally identical pronuclei and with the correct alignment of nucleolar precursor bodies in the regions of interpronuclear contact (Tesarik *et al.*, 2001). This suggests that reprogramming of cumulus cell nuclei has at least started in this system. The complete ultrastructural

remodelling of somatic cell nucleoli after injection to metaphase II oocytes observed in the mouse model (**Figure 2**) also argues in favour of this concept.

The achievement of complete nuclear reprogramming will also depend on a number of epigenetic factors, including the quality of the oocyte activation stimulus and methylation reprogramming of the gamete-derived and the somatic cell-derived genome. In this context, it is clear that researchers will be confronted with biologically quite different situations when using haploidized somatic cell nuclei in the treatment of female and male sterility. The former is closer to the physiological condition, because the oocyte activation signal is delivered by spermatozoa, whereas in the latter case, recourse to an artificial activation stimulus is needed. Moreover, the paternal and the maternal genomes show unequal methylation/demethylation dynamics in early mouse embryos, and these are different from cloned embryos (Dean *et al.*, 2001). For instance, the paternal genome undergoes extensive genome-wide demethylation in the 1-cell zygote, while imprinted genes are protected against this demethylation; the possible lack of protection of the differential methylation pattern in imprinted genes during this early post-fertilization demethylation wave has been discussed in relation to the presumptive alteration of imprinting patterns in cloned animals (Dean *et al.*, 2001). The maternal genome, in contrast, does not undergo demethylation in the zygote (Dean *et al.*, 2001). Hence, zygotes resulting from syngamy between an oocyte- and a somatic cell-derived nucleus are likely to be at higher risk of methylation and imprinting abnormalities as compared with zygotes resulting from syngamy between a sperm- and a somatic cell-derived nucleus.

Regarding other epigenetic factors, the problem of co-existence of a somatic cell-derived and a sperm-derived centriole in fertilized reconstructed oocytes has been discussed recently (Tesarik, 2002).

In conclusion, most of the problems related to the development of somatic cell haploidization procedures are clearly defined and easy to address experimentally. Together with the enormous therapeutic potential that this approach might have in the future, this situation should encourage further intensive research focused on individual open questions.

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