Reproductive semi-cloning respecting biparental embryo origin

Embryos from syngamy between a gamete and a haploidized somatic cell

Jan Tesarik

Laboratoire d’Eylau, 55 rue Saint-Didier, 75116 Paris, France and Molecular Assisted Reproduction and Genetics, Gracia 36, 18002 Granada, Spain

3Correspondence should be addressed to: Laboratoire d’Eylau, 55 rue Saint-Didier, 75116 Paris, France.
E-mail: rtesarik@hotmail.com

Embryos formed by somatic cell nuclear transfer to enucleated oocytes (cloning) have given rise to viable offspring in several mammalian species. The possibility of future application of this technique to human assisted reproduction (reproductive cloning) has been widely debated. On this background there is current discussion of the potential for a cloning-derived technique, which aims at syngamy between a gamete nucleus from one parent and a somatic cell nucleus from the other. Critical analysis of the clinical indications, the current state of the art, biological concerns and ethical considerations relative to this technique, called here reproductive semi-cloning, are presented. Such a technique requires validation by further research before it can be considered as a treatment option. This debate explores issues raised by the technique.

Key words: assisted reproduction/cloning/gamete reconstruction/haploidization

Introduction

Recent progress in mammalian cloning technology (Wakayama and Yanagimachi, 2001a; review) has led to considerations about the potential use of cloning in human reproductive medicine (Abbott, 2001; Nielsen et al., 2001; Pickrell, 2001). The prevailing feeling was that the application of cloning in assisted reproduction is still premature nowadays because of low efficacy and uncertainties about the potential health hazards for the offspring (Bonnicksen, 2001; Soules, 2001). Moreover, the expected end product of cloning is an embryo whose nuclear genome is entirely derived from a single individual. Cloning is thus not well-suited for the solution of infertility problems because of the uniparental origin of the resulting progeny.

The total and definitive lack of developmentally competent gametes, which is the main motivation for the use of the patient’s somatic cell as the source of his/her genetic contribution to the future child, does not usually concern both partners in an infertile couple simultaneously. Hence, the development of techniques allowing the formation of embryos containing a blend of the gamete genome, originating from one parent, and the somatic cell-derived genome from the other parent would certainly be better suited for assisted reproduction purposes than conventional cloning (Kubiak and Johnson, 2001).

In the past 2 years the combined use of gametes and somatic cells for embryo formation has been attempted in several laboratories. Preliminary experience from these attempts suggests that, when combined with the gamete of the opposite sex, different types of somatic cells may be useful as substitutes for both the female (Tesarik et al., 2001) and the male (Lacham-Kaplan et al., 2001) gamete.

In this paper the basic clinical, biological and ethical aspects of this newly emerging technique are discussed, and the main differences from conventional cloning are outlined. This is intended as a basis for a broader debate among specialists which, in its turn, is expected to provide elements for law-makers and institutional review boards involved in decision-making deliberations as to further development and application of this technique.

Clinical indications

The combined use of a gamete and a somatic cell in assisted reproduction was originally suggested as a means for reconstruction of new oocytes consisting of an artificially haploidized somatic nucleus and an enucleated oocyte from a donor; these oocytes need to be fertilized by a spermatozoon to form an embryo (Tsai et al., 2000; Tesarik et al., 2001). This would enable genetic motherhood to women who lack ovaries because of an inherited developmental anomaly or whose ovaries had to be removed for various pathological conditions, but also to those who do have ovaries but whose oocyte reserve has been irreversibly lost as a result of cancer therapy, precocious menopause or other diseases.
Recent data have suggested that somatic cells can also be used as substitutes for the male gamete (Lacham-Kaplan et al., 2001). The technique may thus become the only possible treatment conserving the genetic parenthood of men with a total lack of germ line in the testes. In testiculopathies in which the germ line is present but sperm are not formed because of maturation arrest at different stages of meiotic and post-meiotic development, the technique would represent an alternative to other, still largely experimental treatments involving the injection of immature germ cells into oocytes (Tesarik et al., 1995; Sofikitis et al., 1998) or germ cell in-vitro maturation (Tesarik et al., 1999).

**Biological concerns**

The technique whereby the embryonic nuclear genome is created as a blend of the gamete genome from one partner with the somatic cell genome from the other raises the same concern as conventional cloning. The health of the resulting child may be threatened by dire consequences of chromosomal abnormalities, due to defective chromosome segregation during somatic nucleus haploidization, inadequate nuclear reprogramming, excessively short telomeres, genomic imprinting abnormalities and the co-existence of gamete and somatic cell centrosomes.

**Fidelity of chromosome segregation during somatic nuclear haploidization**

Unlike conventional cloning, reproductive semi-cloning required a reduction of somatic cell nuclear DNA content to a half in order to obtain a diploid embryo after syngamy with the gamete nucleus. Preliminary experiments suggest that this task might be realized by the metaphase II oocyte whose cytoplasm is capable of forcing G0/G1 somatic cell nuclei to a premature M-phase without previous S-phase (Figure 1), resulting in segregation of one set of single-chromatid chromosomes to a pseudo-second polar body (Tesarik et al., 2001). Fluorescence in-situ hybridization (FISH) analysis of human pseudo-second polar bodies extruded after ICSI to human oocytes reconstructed from cumulus cells showed that segregation of single-chromatid chromosomes actually took place (Tesarik et al., 2001). However, this analysis was performed for five chromosomes only (chromosomes 13, 18, 21, X and Y), and it gave interpretable results only in two out of three pseudo-second polar bodies extruded from three zygotes that were subsequently cryopreserved for eventual future transfer. Confocal microscopy analysis of seven mouse oocytes fertilized with haploidized cumulus cells also showed the separation of cumulus cell chromosomes into two sets and the extrusion of one of these sets in a pseudo-second polar body (Lacham-Kaplan et al., 2001), but no FISH analysis was performed in that study. The fidelity of somatic nucleus chromatid segregation into two sets and their distribution between the oocyte and the pseudo-second polar body thus remains to be evaluated. This evaluation needs to be performed in an appropriate animal model before definitive confirmation with human oocytes, since the availability of good quality human oocytes for this kind of research will always be limited.

The ooplasmic activity capable of forcing G1 single-chromatid chromosomes to a premature metaphase with subsequent segregation of the two parental chromosomal sets appears to be only present in metaphase since the injection of somatic cell nuclei at any phase of the cell cycle to prophase (germline vesicle) mouse oocytes resulted in a chaotic behaviour of chromatids and chromosomes (J. Fulka Jr, personal communication). Metaphase II oocytes thus appear to represent the optimal vehicle for somatic nucleus haploidization.

Nonetheless, the way somatic nucleus haploidization is proposed to take place in metaphase II ooplasm is quite different from what happens during meiosis in the germ line (Figure 1). In normal meiosis reductional segregation occurs at meiosis I, when two replicated, homologous chromosomes that are physically attached by chiasmata at sites of chromosome exchange during crossing over, segregate from each other. At meiosis II oocytes therefore contain one set of chromosomes with two chromatids each. Except for exchanged parts, the two chromatids of each metaphase II chromosome are thus either paternally or maternally inherited (Figure 1). This situation is not comparable with that resulting from the introduction of G1 chromosomes to metaphase II ooplasm. Here, two sets of single-chromatid chromosomes originating from both parents co-exist (Figure 1).

Unlike metaphase II chromosomes in normal meiosis, when the two chromatids of each chromosome are still physically attached to each other at their centromere, homologous single-chromatid chromosomes derived from G1 somatic nuclei lack any physical association. This lack of cohesion may increase the risk of errors during the second meiotic division. In fact, premature loss of cohesion at centromeres (premature chromatid separation) during prolonged metaphase II arrest (e.g. during postovulatory ageing) has been shown to increase the frequency of aneuploidy in embryos (Mailhes et al., 1998). Further analysis of the fidelity of somatic nucleus-derived chromosome segregation at anaphase II is thus required to evaluate the risk of aneuploidy associated with the use of somatic nucleus haploidization in reproductive medicine.

**Nuclear reprogramming**

As compared with conventional cloning, the combined use of a gamete and a somatic cell to form an embryo represents a kind of semi-cloning, the success of which will depend on the extent of somatic nucleus remodelling before entering syngamy with the gamete nucleus. The cytoplasm of oocytes at metaphase of the second meiotic division appears to possess unique capacities to realize this complex nuclear rearrangement. So, human cumulus cell nuclei which were driven to premature M-phase after injection to human metaphase II oocytes and which remained in this stage for 14 h subsequently formed pronuclei with apparently normal morphology (as judged by examination in the living state in an inverted microscope equipped with interference contrast optics) instead of resuming the typical morphology of the cumulus cell interphase nucleus (Tesarik et al., 2001). The cumulus cell-derived ‘pseudo-female’ pronucleus and the sperm-derived male pronucleus established a close contact and underwent syngamy followed by the first embryonic
Normal meiosis | DNA content | Artificial haploidization
---|---|---
Oogonium | 4C | G0/G1 somatic nucleus
Last pre-meiotic mitosis | | Metaphase in ooplast (skipping S-phase)
Primary oocyte | 2C | Reconstituted oocyte
S-phase | | Activation by sperm
Primary oocyte | 4C | Activated oocyte
First meiotic division | | Syngamy
Secondary oocyte | 2C | Zygote
Activation by sperm | | S-phase
Activated oocyte | 1C | Zygote
Syngamy | | Metaphase in ooplast (skipping S-phase)
Zygote | 2C | Reconstituted oocyte
S-phase | | Activation by sperm
Zygote | 4C | Activated oocyte

Figure 1. Schematic representation of cell cycle events occurring during artificial somatic cell haploidization for oocyte reconstruction, as compared with normal meiosis. Only one chromosome is represented. Vertical bars represent individual chromatids. The letter above each represented chromosome indicates its paternal (P) or maternal (M) origin. Sperm-derived chromosome set (S) complementing the oocyte derived set in the zygote is also represented. Attachment of sister chromatids at centromeres is represented by horizontal bars between individual chromatids. The cross between homologous chromosomes symbolizes the presence of exchange/chiasma sites. In different stages of normal meiosis and of artificial haploidization the represented chromosome occurs in two versions or only in one version, each version consisting of one or two chromatids. In both normal meiosis and artificial haploidization the haploid status is achieved after oocyte activation by the fertilizing spermatozoon so that the set of single chromatids of maternal origin can be complemented by a sperm-derived chromatid set (S) to restore the normal chromosome number after the subsequent first zygotic S-phase. In normal meiosis reductional division occurs at meiosis I and results in the elimination of either the paternally derived or the maternally derived chromosome set with the exception of chromosome parts exchanged at chiasmata during crossing over. On the other hand, a complete (no crossing over) elimination of either paternally or maternally derived chromosomes occurs only at anaphase II, in activated oocytes, during artificial haploidization. Adapted from Tesarik et al. (2001) Reprod. Biomed. Online, 2, 160–164.

cleavage division by 40 h after sperm injection into the reconstructed oocytes (Tesarik et al., 2001). A similar synchronous development was reported for somatic cell-derived and oocyte-derived pronuclei when the somatic cell (cumulus cells or fibroblasts) nuclei were injected into metaphase II mouse oocytes to substitute for the male gamete after subsequent oocyte activation by ethanol (Lacham-Kaplan et al., 2001). The higher speed of pronuclear development (the first appearance of pronuclei within 2 and 3 h after oocyte activation) observed in the latter study (Lacham-Kaplan et al., 2001) as compared with human semi-cloned zygotes (Tesarik et al., 2001) corresponds to the known differences in the first embryonic cell cycle timing between the two species.

It is possible that additional nuclear reprogramming occurs during pronuclear apposition and syngamy whereby the gamete-derived pronucleus may help remodel the somatic cell-derived pronucleus before the two genomes merge during syngamy. Such phenomena have been speculated to occur when mature (metaphase II) human oocytes are fertilized with immature human male germ cells (spermatids), which would
explain the prolonged duration of the syngamy stage before the beginning of the first cleavage division (Tesarik and Mendoza, 1996). Thus, the presence of one gamete nucleus may be beneficial for the correct remodelling of the somatic nucleus during reproductive semi-cloning as compared with conventional cloning. This hypothesis, however, still remains to be tested, and preliminary experimental data do not speak in its favour. In fact, only nine (17%) of mouse oocytes fertilized by haploidized cumulus cells and two (22%) of those fertilized by haploidized fibroblasts developed to blastocysts in culture, and none of 20 embryos produced by cumulus cell injection and transferred to foster females developed to live pups (Lacham-Kaplan et al., 2001). However, the number of specimens was relatively small in that study, and further modifications of the experimental protocol, including the choice and preparation of the somatic cells to be used, the optimization of the time period between somatic cell introduction to the oocyte and oocyte activation and the methods used for oocyte activation, may improve outcomes. As to the human embryos that were formed by fertilization by sperm of oocytes reconstructed with the use of cumulus cell nuclei, none has been transferred yet, and all will remain frozen until more information about the potential risk to the health of the resulting children is available (Tesarik et al., 2001).

**Genomic imprinting status**

Fetal and placental overgrowth and some other anomalies have been reported in the offspring resulting from culture of mammalian embryos (Khosla et al., 2001) and in cloned animals (Wakayama and Yanagimachi, 2001b), and these anomalies are suspected to be caused by genomic imprinting aberrations. As with conventional cloning, the risk of genomic imprinting abnormalities also represents a major concern for reproductive semi-cloning. This risk may be lower in the latter case because at least one allele of each gene originated from a gamete. Moreover, the imprinting status of the somatic cell nucleus may be subject to a trans-modification activity of germ cell cytoplasm, as demonstrated recently in germ–somatic cell hybrids (Surani, 1999), or may be affected by epigenetic events occurring in the early embryo (Reik et al., 1993, 2001).

**Centrosome and spindle function**

The nucleation of microtubules and the function of the mitotic spindle in early human embryos are dependent on the presence of an active centrosome (complex of two centrioles and pericentriolar material) whose development in mammals, with the only known exception of rodents, is dependent on the sperm-derived proximal centriole introduced into the oocyte during fertilization. At the same time, the oocyte centrosome becomes inactive (Sutovsky and Schatten, 2000). In agreement with this general scheme, the mitotic potential of the human preimplantation embryo has been shown to be paternally inherited (Palermo et al., 1994, 1997; Sathananthan et al., 1996). The presence of two paternal centrioles in the human zygote, occurring after bispermic oocyte penetration, leads to the formation of multipolar mitotic spindles and mosaicism which cannot be corrected by a simple removal of the extra sperm-derived pronucleus (Palermo et al., 1994). Thus, the co-existence of the gamete- and somatic cell-derived centrioles in the embryos resulting from fertilization with haploidized somatic cells raises concern as to the normality of the subsequent mitotic divisions with regard to chromosome segregation at anaphase and ploidy of the daughter cells.

Even though this concern must be taken into consideration, it has to be noted that, unlike the presence of two equivalent (i.e. sperm-derived) centrioles, the co-existence of two non-equivalent centrioles in fused cells usually leads to the dominance of one and the functional silencing of the other (Manandhar and Onishchenko, 1995). Different cell cycle phases of each of the fused cells can be at the origin of this non-equivalence. In agreement with these observations on somatic cell fusions, non-proliferating thymocyte nuclei (G0 phase) introduced into metaphase II mouse oocytes entered metaphase, while their associated centrioles became inactivated and were not involved in spindle formation (Szollosi et al., 1986). This system was very similar to that used in the somatic nucleus haploidization experiments referred to in this article. However, the behaviour of somatic cell-derived and sperm-derived centrioles in reconstructed oocytes and embryos warrants further study. If necessary, the activity of the sperm-derived centriole can be disrupted artificially by mechanical separation of the sperm tail before oocyte fertilization by isolated sperm head injection (Palermo et al., 1997).

**Ethical considerations**

As compared with conventional cloning, in which the new individual is genetically identical (except for a few extranuclear genes) with only one parent, the formation of embryos by syngamy between a gamete and a pseudo-gamete derived from a haploidized somatic cell respects the natural mode of bisexual reproduction. In the case of oocyte reconstruction from a somatic cell nucleus (Tesarik et al., 2001) the newly formed oocyte is subsequently fertilized by injecting a spermatozoon in a quite similar way as ICSI to naturally grown oocytes. The sequence of events is slightly less natural when somatic cells are used to substitute for the male gamete (Lacham-Kaplan et al., 2001). In fact, embryo formation in this latter situation occurs in two phases: the introduction of the somatic nucleus, and the subsequent artificial activation of the oocyte triggering embryo development. The classical concept of fertilization, in terms of an action combining the introduction of the male genome to the oocyte with oocyte activation, is completely absent here.
What about the genetic relationship between the child resulting from the assisted reproduction treatment by conventional cloning or reproductive semi-cloning and his or her parents? As compared with conventional cloning, which simply gives rise to an individual resembling an identical twin of one parent, the genetic constitution of children conceived by reproductive semi-cloning represents a more complicated issue. The participation of that parent who contributes a gamete to the future embryo is the same as in normal fertilization, including the unpredictability of the recombination pattern mediated by meiotic crossing-over. As to the other parent, his/her genetic contribution would be identical to that received from one of his/her own parents. The other grandparent’s contribution would be eliminated completely during the extrusion of one uniparental set of single-chromatid chromosomes to the pseudo-second polar body (Figure 1). This is similar to normal fertilization except for the fact that the elimination of one grandparent’s genome is total because there has been no exchange between homologous chromosomes. It remains to be determined whether this single-chromatid chromosome segregation is random or non-random with regard to the parental origin of that set of chromosomes retained in the oocyte and that extruded to the pseudo-second polar body. Anyway, the technique, if successful, would not only ensure the biparental genetic contribution to the couple’s progeny but, similar to normal fertilization, would also generate a new and unpredictable combination of genetic traits inherited from both parents, which makes it impossible to be abused for the creation of individuals with on-command genetic constitution.

Conclusion

Preliminary evidence suggests that embryos might result from syngamy between a gamete nucleus and a haploidized somatic cell nucleus and that the somatic cell may replace either the male or the female gamete, which makes this technique potentially applicable in the treatment of both male and female infertility. The validity of haploidization is controversial and will require further research. Though sharing some features with conventional cloning, the technique is biologically closer to normal fertilization because the future individual results from the union of two parental genomes of which one is actually brought by a gamete. This would alleviate some of the ethical concerns raised against the use of conventional cloning in human assisted reproduction. At this time, research into the validity of haploidization is necessary and clinical application cannot be considered but it is important that the issues involved are debated.

References