—Mini Review—

Manufacturing and Regenerating of Gametes

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Abstract: *The decline of human fecundability stems from compromised quality and reduced quantity of gametes. The age-related female infertility and spermatogenic failure have been the major obstacle to overcome in ART with only limited success unless donated gametes are used. I review the different approaches to manufacturing and regenerating gametes. Attempts of nuclear transplantation for preventing oocyte aneuploidy thereby enhancing the developmental competence or for inducing haploidization of somatic cell nuclei aiming to generate oocytes and spermatozoa are explored by highlighting advantages and limitations of these strategies. In addition, approaches to differentiate precursor cells or pluripotent stem cells to their progenies or mature gametes are revisited. Finally, I also report preliminary data on enhancing the reproductive performance of a single spermatozoon by male genome cloning utilizing micromanipulation techniques.*

Key words: *Ovarian Failure, Testicular Failure, Nuclear Transplantation, Gametogenesis, Stem Cells*

Introduction

The decreased fertility of older women stems in large part from a decline in the frequency of intercourse, in the number of primordial follicles, and particularly, from a higher incidence of oocyte aneuploidy [1–4]. The likelihood of conception decreases inversely with maternal age, reaching a nadir as early as 40 yr of age [1]. The reason for this resides primarily in the status of the egg and the conceptus rather than in the endometrium, as demonstrated by the higher pregnancy rates in older women receiving donor oocytes [5]. In the arrested MII oocytes, the frequency of aneuploidy, primarily the result of a nondisjunction of bivalent chromosomes occurred during meiosis I, is estimated to be 4.9% in the 25–34, 11.5% in the 35–39, and 29.8%, in the 40–45 yrs age groups [2]. In addition, there have been sister chromatid predivision also responsible for age-related oocyte aneuploidy. Oocyte aneuploidy is not only the major reason for the decreased pregnancy rates in older women, but also a confounding factor for the higher incidence of autosomal trisomy observed in offspring [6].

Attempts to improve the chances of pregnancy in women who are at increased risk for oocyte aneuploidy currently include the selection of genotypically normal oocytes and embryos by the controversial preimplantation genetic diagnosis and selection [7, 8], sometimes complemented by chromosomal assessment of the spermatozoa [9]. Two logical ways of preventing oocyte aneuploidy would be the cryopreservation of oocytes while women are at their young fertile age [10, 11], or even, as some case reports suggest, the cryostorage of the surgically isolated ovarian cortex [12–14].

It has been suggested that the transfer of the nucleus from a germinal vesicle (GV) stage oocyte isolated from an older woman into the ooplast of a younger one may grant a correct progression through meiosis during oocyte maturation [15, 16]. In this circumstance, nuclear transplantation is carried out prior to the occurrence of putative abnormal segregation of chromosomes. The younger cytoplasm would ensure the correct chromosomal segregation resulting in an euploid oocyte by the provision of a functional spindle.

Spermatogenic failure similarly is the major responsible factor for the male counterpart affecting a considerable proportion of couples who fail to conceive by ART. The use of donor spermatozoa has been the only successful treatment in such cases, as egg donation has been for ovarian failure. Azoospermic patients can now be treated by ICSI with spermatozoa isolated directly from seminiferous tubules [17, 18]. However, only in up to 60% of testicular sampling yields spermatozoa while in the remainders spermatogenic arrest, often at the sper-

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matocyte stage, or even germ cell aplasia [19].

The spur for treating dysfunctional or absent gametes has steered investigators to explore neo-gametogenesis, or alternative sources of spermatozoa and oocytes for infertile couples seeking their own genetic child. Moreover, several leads in cloning science have opened routes, real or theoretical, for safe ways to 'manufacture' gametes aiming at making the goal possible. More recently, in-vitro differentiation of embryonic stem cells (ESC) into gamete-like cells has been carried out [20–22], and in one study [23] even generated offspring. More recently, it was reported that fertile spermatozoa have been differentiated from ES and iPS cells [24].

Here, I review the different approaches to manufacturing and regenerating gametes. I revisited the experimental attempts to achieve this goal by nuclear transplantation, aiming at correcting oocyte aneuploidy, or inducing haploidization of somatic cells into oocytes and spermatozoa, describing their advantages and limitations. I also report preliminary data on maximizing the developmental potential of a single spermatozoon as well as the current status of the *in-vitro* culture of spermatogenic cells derived from early maturational stage or embryonic and induced pluripotent stem cells.

Experimental Attempts

Oocytes

1. Nuclear transplantation of immature oocytes

It was suggested by Zhang *et al*. [16] that the transfer of a GV from an aged oocyte into a younger ooplast might represent an additional approach to prevention of aneuploidy. For this, nuclear transplantation needs to be performed at the GV stage prior to the segregation of chromosomes. The younger cytoplasm provides substantial molecular element (s) to support the formation of a healthy spindle, thereby allowing normal chromosomal segregation during meiosis [25, 26]. Since mitochondrial dysfunction can lead to oocyte aneuploidy [27–31], mitochondrial damage was induced as a means of generating dysfunctional ooplasm comparable to the cytoplasm deterioration observed in aging oocytes [26, 32].

Ooplasmic damage inflicted on mouse oocytes at the GV stage apparently inhibited nuclear maturation (6.0%) compared to unexposed controls (85.6%, $P < 0.001$). GV karyoplasts isolated from the affected oocytes and transferred subzonally into intact ooplasts restored maturation (76.2%). Once inseminated, 65.8% were fertilized normally, and 21.1% developed to blastocysts. The few oocytes that were not corrected but matured, fertilized only at a 16.7% rate ($P < 0.001$). Embryos originated from the 'rescued' oocytes developed full-term offspring by transferring to pseudo-pregnant females [32].

This proves that replacement of an ooplasm with extrinsic damage can be successfully corrected by nuclear transfer, allowing fertilization and embryonic cleavage comparable to the intact control oocytes. This intervention is also capable of supporting a correct chromosomal segregation during meiosis I as proven by a limited experience carried out on human oocytes [25, 26].

2. Techniques attempting to coerce chromatinic haploidization

Although GV transplantation has been successful in producing some animal offspring, its overall efficiency remains relatively low, mainly due to the limited availability of oocytes as well as the requirement of *in-vitro* maturation [15, 32–34]. In fact, oocytes of older women are not just chromosomally compromised, but they are also in short supply. Thus, even an optimized GV transplantation would depend on the number of eggs available.

A more radical approach would be the generation of a *de novo* gamete [35, 36]. To produce gametes, precursor germ cells undergo meiotic division resulting in haploid cells. Preliminary experiments showed ooplasm is capable of initiating a meiosis-like reductive division of a somatic cell nucleus [26, 36, 37]. Encouraging findings involving haploidization of a somatic cell were reported from other laboratories [38, 39]. While this approach appears similar to cloning, the resulting pseudo-gametes need the participation of the paternal genome to produce a biparental progeny.

Enucleation of GV oocytes and subsequent somatic cell grafting was accomplished in 98% of attempts, with 72% being reconstituted successfully by electrofusion. Subsequently, 51.8% of the constructs extruded a 'pseudo first polar body (PB)' following a 14–16 h culture period. A 38.5% of the analyzable karyotypes of oocytes that extruded a PB showed having a set of haploid number of chromosomes in the ootid and a PB, while the large majority showed numerical and structural chromosomal aberrations such as aneuploidy, pulverized chromosomes, and diploidy in the telophase. The dysfunction of this induced pseudo meiotic process involves an absent recombination and an inability for assembling a reliable kinetochore-spindle fibers complex [40].

This phenomenon is probably due to the inability of an immature cytoplasm to reprogram the somatic nucleus by driving them into an M phase while bypassing DNA synthesis. Utility of an MII ooplast provided 62% of reconstitution rate, however, in an experiment failed to extrude a PB generating an additional pronucleus at a

rate of 17% [41]. Haploidization of cumulus cells within enucleated human oocytes was confirmed by FISH analysis, although with a limited number of chromosomes assessed, on either the second polar bodies or activated bipronuclear oocytes [25, 38, 41]. Pronuclei (PNs) derived from somatic cell injection proved to be 'putatively haploid' in approximately 38% of cases when chromosomes 13, 14, 15, 16, 18, 21, 22, and X were assessed [41]. Thus somatic cell nucleus 'haploidization' can take place both in immature and mature ooplasm, with the latter requiring oocyte-activating stimuli. However, the odds of obtaining a normal haploid complement of chromosomes in an oocyte undergoing its second meiotic division after somatic cell nuclear transplantation prove to be limited [41–45]. Correct chromosome segregation is crucial in artificial haploidization. Meiotic chromosomes in an oocyte display a behavior different from that of the mitotic chromosomes in the somatic cell. In meiosis, the MII chromosomes consist of two chromatids which are physically attached to each other at their centromere, while G_0/G_1 cumulus cells contain monovalent chromosomes. The correct position and attachment of the chromosome on the spindle, as well as a distinctive regulation of the cohesion between sister chromatids seems to be crucial for correct chromosome reduction. When G_0/G_1 somatic chromosomes are transferred into MII ooplasm, there is no physical association between their homologous single chromatids. In the absence of any cohesion at all, reduction division should totally be random [41, 44].

3. Induced neo-differentiation of oocytes

It has been demonstrated that embryonic stem cells (ESCs) are capable of differentiating into all three germ layers (endoderm, mesoderm, and ectoderm) of the embryo proper as well as germ cells *in vivo* by chimera production and tetraploid complementation [46, 47]. Maintaining ESCs in monolayer cultures, sporadic oocyte-like structures have been identified that presumably develop into a structure resembling a blastocyst [20]. Follicle-like structures were also obtained by feeding embryo bodies (EBs) with conditioned medium isolated from cultures of mouse neonatal testicular tissue [48]. These structures were not, however, capable of developing further. Human ESCs also are capable of differentiating into cells expressing germ cell-specific genes [49]. Some investigators reported derivation of oocyte-like cells from mouse ESCs, however, the full characteristics of these cells as female gametes have not been elucidated yet [20, 48]. In fact, ES-derived oocyte-like cells undergo spontaneous activation and lack zona pellucida protein 1 [20]. Recently, induced pluripotent stem cell (iPS) technology [50–52] has been proposed as an alternative for therapeutic cloning. Although oocyte differentiation from iPS cells has not been attempted or reported yet, oocytes originated from somatic cells with an identical genome may possibly be generated, providing further options of utilizing stem cell-derived oocytes.

Spermatozoa

1. *In-vitro* spermatogenesis

In mammals, millions of spermatozoa are produced daily, ultimately from spermatogonial stem cells (SSCs). These precursors of spermatogonia depend for their survival and proliferation on specific growth factors, and on a close relationship with enveloping presumptive Sertoli cells that seems prevent the apoptosis of these germ cells. The ability to propagate and immortalize SSCs *in vitro* would allow the creation of colonies which, on intratesticular transfer, might be able to repopulate the germinal epithelium in azoospermic men with Sertoli-cell-only syndrome.

We have attempted to establish methods for selection, proliferation, and eventual maturation of mouse SSCs in a serum-free culture system. In order to distinguish differentiating germ cells, we employed alkaline phosphatase (AP) activity, Thy-1 presence, and VASA expression as markers for the identification of primordial germ cells (PGCs), SSCs, and the spermatogenic cells, respectively, while FE-J1 and Scp1 were employed for assessment of the post-meiotic stages.

Sorting of dissected testes from 6-day-old neonatal mice by magnetic separation yielded Thy-1 positive cells with a >80% enrichment rate ($P < 0.0001$). After plating the sorted germ cells on a feeder layer, one putative SSC colony, confirmed as such by specific markers, proliferated up to day 9 of culture in a serum-free culture condition. On the other hand, the tridimensional support provided by testicular somatic cells along with essential growth factors such as GDNF, bFGF, and LIF, assured the proliferation and propagation of germ cells for an extended time (more than 50 days). VASA expression and AP activity were present mainly on the periphery of the aggregates and on some individual cells within, indicating that the germ cells were maintained for an extended time. Following administration of FSH and LH, it became possible to identify post-meiotic cells [53, 54].

Very recently, complete in-vitro spermatogenesis from neonatal spermatogonia and even cultured SSCs, in an organ culture system, was accomplished in mice [55, 56].

2. Spermatogenic cell transplantation

The continuation of the spermatogenic process

throughout life relies on the proper regulation of self-renewal and differentiation of spermatogonial stem cells. These cells situated on the basal membrane of the seminiferous epithelium represent only 0.03% of the germ cell population. They are the only cell type that can repopulate and restore fertility to congenitally infertile recipient mice following transplantation. Although numerous expression markers such as THY-1 and GFRalpha-1, and absence of c-kit, have been helpful in isolating and enriching spermatogonial stem cells, no specific marker for this cell type has yet been identified. However, much effort has been directed toward the maintenance of spermatogonial cells *in vitro*, and recently, co-culture systems of testicular cells on various feeder cells have made it possible to culture spermatogonial stem cells for a long period of time, as demonstrated by the transplantation assay [57].

In fact, in animal, when spermatogonial stem cells were isolated from infertile testes and transplanted into host seminiferous tubules, viable mature spermatozoa developed and live offspring were obtained [58]. Thus, once techniques to isolate and propagate spermatogonia *in vitro* are established, those (e.g. cancer patients) who want to preserve their fertility may be treated by autologous spermatogonia transplantation. At our laboratory, we investigated whether it is possible to induce human spermatogenesis in host testes by transplanting spermatogenic cells obtained from testicular biopsy specimen into mouse seminiferous tubules (xenogeneic transplantation). Although testicular cells were successfully injected into the seminiferous tubules, neither proliferation of SSCs nor spermatogenic differentiation was observed [59]. So far, this procedure has worked only under autologous conditions [60].

3. Techniques attempting to coerce male somatic cell haploidization

As described in the female section, it has been attempted to generate male haploid gametes and zygotes by utilizing the technique that involves ooplasmic somatic cell haploidization [39, 61]. At our laboratory, we assessed the ability of ooplasm to induce the haploidization of male somatic nuclei. As a source of somatic cells, single mouse male fibroblasts, prepared by culturing minced skin tissue, were utilized. Intact mouse MII oocytes were injected with single fibroblasts, and were then incubated for at least 2 h and subsequently exposed to 10 mM $SrCl_2$ for 6 h to induce oocyte activation. Haploidization occurrence and fertilization were confirmed by observation of two distinct PNs and simultaneous extrusion of two PBs. A total of 155 (91.7%) among 169 MII oocytes survived male fibroblast injection. After oocyte activation, 74 (43.8%) displayed two PNs and two PBs. Subsequently, 70 (41.4%) of these constructs underwent first embryonic cleavage and finally 13 (7.7%) reached the blastocyst stage. Preliminary cytogenetic assessment evidenced a 9.5% (4/42) normal numerical chromosomal distribution. Thus, we found that although injected fibroblast nuclei undergo chromosomal condensation and form meiotic spindle-like structure resembling the metaphase II plate, and segregate into two groups, one as a 'male pronucleus' and the other as a 'PB', chromosomal segregation is inconsistent for the same reason as in female somatic cell haploidization.

4. Male genome cloning

The scarcity of gametes often represents a main hindrance to overcoming spermatogenic failure by the use of assisted fertilization techniques. Thus, the possibility to propagate a male genome would provide an alternative means through which to consistently obtain conceptuses. The original concept was to duplicate sperm genome for genetic assessment prior to fertilization [62], and this was recently reproduced with human oocytes [63]. We further extended the idea and attempted to replicate a haploid male genome and generate conceptuses capable of undergoing full term development aiming at enhancing the reproductive performance of a single spermatozoon [54, 64, 65].

Of the intact mouse MII oocytes initially manipulated, 87.5% survived enucleation and single sperm injection, and all came to display a single male pronucleus. A large majority (92.3%) entered cleavage and maintained their haploid status in approximately 90% of the analyzed pseudo-blastomeres. Karyoplasts isolated from cleaving haploid androgenotes were transferred subzonally to haploid parthenotes displaying a single pronucleus, generated by exposing to $SrCl_2$. Once constructs were electrofused to generate biparental zygotes (nuclear transfer fertilization; NT-fertilization) and during the following 4 days of culture, 77.4% of the study constructs developed into blastocysts at a rate comparable to the ICSI embryos (81.1%). The transfer of 64 blastocysts to the uterine horns of 6 pseudo-pregnant females yielded 11 offspring. Utilizing such technique (Fig. 1), we obtained so far live offspring with 8-cell stage androgenotes. Thus we have demonstrated 'the proof of principle' in empowering single spermatozoa with male genome cloning.

5. Neo-differentiation of spermatogenic cells

As described earlier in the female section, ESC differentiation into spermatogenic cells also has been report-

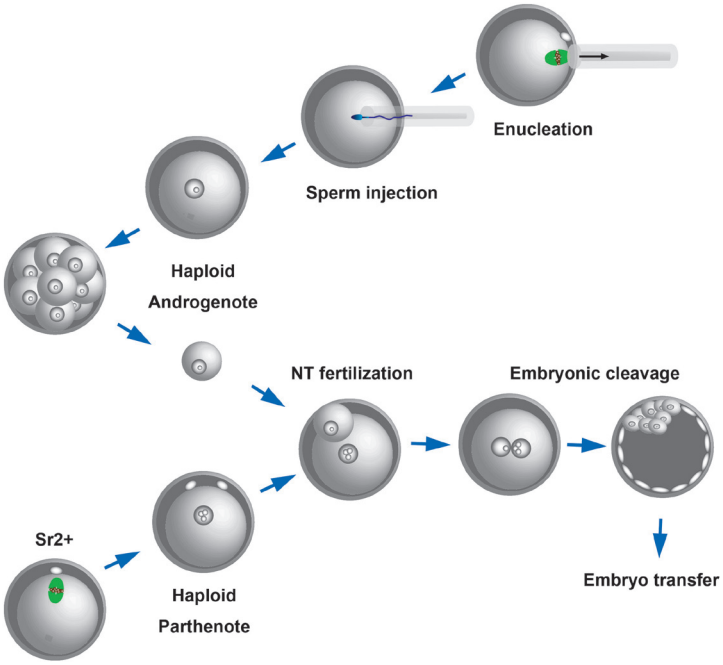

Fig. 1. Male genome cloning. To generate a haploid androgenote, a metaphase II oocyte is enucleated and injected with a single spermatozoon. A haploid androgenote displaying one pronucleus is cultured to allow embryo-like cleavage. Each blastomere maintains its original haploidy. A karyoplast is isolated from a single blastomere of a haploid androgenote, while a haploid parthenote is generated by exposing an intact metaphase II oocyte to $SrCl_2$. A biparental zygote is reconstituted by nuclear transfer-fertilization where an androgenetic karyoplast is electrofused with a parthenote, and cultured up to the blastocyst stage and transferred to a pseudo-pregnant female.

ed [21–23]. The derivation of male gametes from ESCs involves the formation of EB, and the isolation of putative PGCs, precursor cells of gametes, from the EB. To enhance germ cell differentiation, above earlier studies have suggested the addition of bone morphogenetic protein (BMP) into the culture system. Enhancing the number of germ cells present increases the chances of them differentiating into later post-meiotic stages.

Recently, mouse offspring were obtained after fertilization with spermatozoa differentiated from ES cells [23]. However, the resultant offspring from ES-derived spermatids were abnormal due to imprinting errors [23]. More recently, it has been demonstrated that both mouse ES and iPS cells differentiate into fully fertile spermatozoa via primordial germ cell-like cell derivation although requiring *in-vivo* spermatogenesis in a host seminiferous tubule [24].

Future Directions and Conclusions

Nuclear transplantation itself does not increase the incidence of chromosomal abnormalities, since mouse and human oocytes reconstituted with homologous donor GVs resume meiosis to metaphase II and maintain a normal ploidy [15, 25]. It is possible to successfully induce photosensitization-based damage in mouse oocyte mitochondria which consistently inhibits GV breakdown, meiotic spindle formation, chromosomal segregation and PB extrusion. Thus, such oocytes can serve as a model in which to study the age-related ooplasmic dysfunction seen in humans. It was also demonstrated that GV transplantation enables such 'rescued' oocytes to undergo maturation, fertilization, embryonic cleavage and ultimately develop to term [32]. GV transplantation has proven to be a highly efficient procedure also in mice, in that >90% of reconstituted oocytes are able to extrude a polar body and display a normal chromosomal constitution. With human oocytes, however, lower maturation rates have been the rule, probably due to the suboptimal procedures currently available for their *in-vitro* maturation. Nonetheless, nuclear transplantation might ultimately provide an attractive approach to treatment of the

age-related aneuploidy seen especially in poor responders and in older patients. The limited availability of human oocytes often makes it difficult to draw firm conclusions. Therefore, a mouse model was designed to simulate the ooplasmic damage of aged human oocytes that involves the selective disruption of the mitochondria. Subsequently the nucleus of the impaired oocyte was rescued by transferring it to a healthy ooplast. This approach has shown in principle that nuclear transplantation can rescue nuclei isolated from a damaged ooplasm, with reasonable efficiency and can generate offspring [32]. However, the limit on the number of human oocytes available still remains a confounding factor for the immediate application of this radical approach to the correction of oocyte aneuploidy in humans.

Oocytes are able to induce 'haploidization' of transplanted somatic cell nuclei independently of the gender of the donor cell. Fertilization of such artificial oocytes was followed by embryonic cleavage even though blastocyst development rates and their chromosomal content are compromised. However, this technique seems unable to provide consistent reliable haploidization of somatic cells due to the lack of proper interaction between the chromosomes and the spindles. It was recently shown that it is possible to replicate the male genome through its injection into ooplasts. Such androgenotes maintain their genotype, ploidy, and the capability of full-term development. The technique further indicates the possibility to create multiple copies of the male genome through which to gain genetic information on a particular gamete or to propagate it when it is scarce.

It has been indicated also the possibility of *in-vitro* neo-differentiation of gametes from ESCs. While these studies suggest that alternative sources of gametes are not merely the stuff of science fiction, but a reality, most of the manipulations involved are unlikely to be applied to man in the very near future. However, while stressing that the genetic normality of the offspring and the safety of the procedures tested in animal experiments must first be firmly established, and the experimental results obtained so far seem to justify further research.

There have been two defined processes with regards to nuclear reprogramming; 1) 'dedifferentiation' is defined as a process where a differentiated cell acquire totipotency, and for example therapeutic cloning and the iPS cell technique [50] fall into this category, 2) 'transdifferentiation' or direct reprogramming is referred to as rather direct switching of one type of differentiated cells to another, therefore, it does not involve ESC or iPS cell derivation. One of our ultimate goals is to identify the factor (s) that can induce 'transdifferentiation' process of somatic cells where conversion of somatic cells to gametes takes place. Understanding such mechanisms certainly requires *in-vitro* gametogenesis, nuclear transplantation and stem cell technologies.

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