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Abstract

Reproductive biotechnology has developed rapidly and is now able to overcome many birth difficulties due to infertility or the transmission of genetic diseases. Here we introduce the next generation of assisted reproductive technologies (ART), such as mitochondrial replacement technique (MRT) or genetic correction in eggs with micromanipulation. Further, we suggest that the transmission of genetic information from somatic cells to subsequent generations without gametes should be useful for people who suffer from infertility or genetic diseases. Pluripotent stem cells (PSCs) can be converted into germ cells such as sperm or oocytes in the laboratory. Notably, germ cells derived from nuclear transfer embryonic stem cells (NT-ESCs) or induced pluripotent stem cells (iPSCs) inherit the full parental genome. The most important issue in this technique is the generation of a haploid chromosome from diploid somatic cells. We hereby examine current science and limitations underpinning these important developments and provide recommendations for moving forward.

INTRODUCTION

Around 10% of couples experience infertility (1). The etiology therein is caused by the female in 40% of cases and the male in another 40%. In 10-20% of cases, both the male and female contribute to the lack of pregnancy success, and unexplained infertility is observed in up to 10% of cases (2). Couples who suffer from fertility issues often use assisted reproductive technologies (ART), such as intrauterine insemination (IUI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (3,4). Since the first birth of an IVF baby in 1978, IVF has been the predominant treatment for female infertility (5). ICSI is an advanced ART wherein a single sperm is introduced into the oocyte through the zona pellucida via microinjection (6). The first successful ICSI was reported by Palermo et al. in 1992; the technique has since become a common treatment for male infertility (7).

Preimplantation genetic diagnosis (PGD) has been used in association with ART to analyze the DNA from embryos at the cleavage or blastocyst stage to determine genetic abnormalities (8). Recent techniques isolate a few cells from the trophectoderm that will become placenta or amnion during embryo development. These isolated cells can be genetically sequenced for disease mutations, and embryos negative for these mutations can then be transplanted into the mother. Multiple rounds of IVF are frequently needed to obtain healthy embryos. ART increases the chance of delivering a healthy baby without heritable genetic disorders caused by known mutations or chromosomal abnormalities (9).

However, it is significantly more difficult to predict the transmission of mitochondrial DNA (mtDNA) mutations by PGD due to asymmetric segregation of mtDNA (10,11). Homozygous nuclear mutations, even rarely-occurring ones, cannot be screened out by PGD because all oocyte or sperm will contain the given mutation, leaving no embryos available for pregnancy. Alternatives are thereby urgently needed to avoid the potential transmission of mtDNA-based disorders or homozygous nuclear mutations.

CURRENT APPLICABLE ASSISTED REPRODUCTIVE TECHNOLOGIES

Mitochondrial replacement therapy (MRT) is a series of processes that involve extracting nuclear DNA with a small amount of cytoplasm from an oocyte or zygote in a patient with mutated mtDNA and then transplanting it into donor cytoplasm where the donor's nucleus has been removed, replacing it with non-mutated mtDNA from the donor (12). Currently, there is no cure for mitochondrial disease, and MRT is the only technology proposed to eliminate the risk of disease inheritance in offspring. Advanced female age is another important cause of infertility, partially due to a cytoplasmic deficiency which induces chromosomal abnormalities in aged oocytes resulting in the failure of fetal development. MRT can also be employed to resolve cytoplasmic defects due to aging (13).

There are several methods for MRT, including pronuclear transfer (PNT), spindle transfer (ST), and polar body transfer (PBT) (Fig. 1). Initially, PN transfer from one zygote to another was performed, and a reconstructed mouse zygote was shown to develop into a live offspring (14). However, a high carryover of mutated mtDNA was detected in the tissues of PNT offspring due to the large amount of cytoplasm that must be physically maintained around the substituted PN structure. Lower levels of mtDNA carryover were shown, however, by performing PNT in human embryo development (15).

ST was pioneered in the monkey and has demonstrated the efficiency and safety of MRT verified by normal growth in the live birth and low levels of mtDNA carryover (16). Based on an ST study in the monkey, human ST was performed using oocytes with different mtDNA haplotypes for mtDNA tracking (17). In ST embryos, embryonic development and ESC establishment rates were similar to control IVF. In ESCs derived from ST embryos, mtDNA carryover levels were undetectable, however, certain haplotype combinations were reverted to maternal mtDNA, meaning 100% carryover (18). The world's first ST baby was

born in Mexico (19). The mother harbored the mtDNA 8993T>G mutation for Leigh syndrome, and ST was carried out in order to avoid the transmission of this disease to her child.

PBT is performed with residual nuclei (PB1 or PB2) not used in PNT or ST and can increase the yield of treated embryos after MRT. During meiosis, mammalian oocytes undergo two reductive cleavages through uneven cytoplasmic separation and two small body abstractions (polar body), harboring chromosomes. The first polar body (PB1) contains diploid chromosomes, and the second polar body (PB2) contains a haploid chromosome. In the mouse, PBT demonstrated normal meiosis in reconstructed oocytes and production of viable offspring (20, 21). In humans, reconstructed PBT oocytes completed for meiosis after fertilization with sperm, while in general, PBT-derived blastocysts gave rise to phenotypically normal ESC lines (22).

However, MRT does involve certain issues. First, a significant increase in the number of healthy egg donors and patient eggs would be required to conduct the necessary research, not to mention its clinical application. Second, poor sperm quality or azoospermia in the partner could limit the application of MRT through ICSI. MRT is also not appropriate for the treatment of nuclear genetic diseases with homozygous mutations, as such mutations will be present in all embryos generated by the MRT.

ASSISTED REPRODUCTIVE TECHNOLOGIES FOR THE FUTURE

Oocytes or sperm can be differentiated from pluripotent stem cells

If oocytes and sperm could be made from adult somatic cells, they could be used for people who suffer from infertility or genetic diseases (23) (Fig. 2). Oocytes or sperm have not yet been effectively produced using human pluripotent stem cells (PSCs). In 2011, Katsuhiko Hayashi and his colleagues showed that sperm and oocytes generated from male or female PSCs enabled

an infertile mouse to have healthy pups for the first time (24,25). Primordial germ cells (PGCs) are naturally generated from epiblast cells, which are developed from ICM after embryo implantation. PGCs migrate back to the fetus and reside in the ovaries or testes, where they develop into eggs or sperm, respectively. In *in vitro* oogenesis, PSCs were induced to develop into epiblast-like cells, which were then induced to become PGC-like cells (26); these PGC-like cells differentiated to primary oocytes. These primary oocytes developed into GV oocytes, which were matured to functional MII oocytes. One limitation therein is that gonad somatic cells are required for the generation of the primary oocyte from PGC-like cells.

This limitation also appeared *in vitro* spermatogenesis where testicular tissue was used for differentiation of sperm from PSCs (27). In humans, spermatids were generated from human spermatogonial stem cells (SSCs) *in vitro* (28). However, complete *in vitro* spermatogenesis starting from human PSCs has yet to be achieved. In *in vitro* oogenesis, oogonia have been induced from human PSCs (29). Ovarian follicle-like cells have also been generated from PSCs overexpressing *DAZL* and *BOULE in vitro* (30).

Although a great deal of mouse research has been performed for oogenesis and spermatogenesis, it is inappropriate to attempt to apply such research directly to humans. Three main factors must be considered before human application. First, the initiating cell type for oogenesis or spermatogenesis is critical. Some researchers reported generation of epiblast stem cells (31-33), but Hayashi and colleagues claimed that these cells did not produce PGCs. Instead, they generated epiblast-like cells from mouse embryonic stem cells (ESCs), which developed into PGCs, and later oocytes or sperm (34). Second, converting PSCs to specific types of cells is challenging, and not all cells respond equally. If PSCs do not differentiate into PGCs, certain cells may differentiate into unwanted cell type instead; as such, purification of PGCs is important to eliminate potential unwanted cell types. Third, to generate functional sperm or oocytes from PSCs, they still must be transplanted into the ovaries or testes, or gonad

cells if they are generated *in vitro* without transplantation (24,26). Identifying and replicating how PGCs mature in the ovaries or testes *in vitro* is crucial for minimizing the number of invasive procedures required for PGCs maturation.

Cure of genetic diseases in germ cells

In the case of a couple carrying a genetic mutation, PGD, which is the clinical standard for treating genetic diseases, is currently the only way to avoid transplantation of mutated embryos. Recently, DNA cutting techniques known as CRISPR/Cas9 (35), have been modified to edit genes in embryos directly. Researchers attempting to edit human embryos using CRISPR have made some progress (36). Several scientific groups in China have reported genetic correction with CRISPR/Cas9 in human zygotes (37,38). Recently, Ma et al. (35) described the correction of a pathogenic mutation in human embryos with CRISPR technology; they corrected the heterozygous *MYBPC3* mutation with high HDR efficiency. Other studies were the first to use a base editor system to correct the *HBB* (A>G) mutation in a human embryonic genome (39,40). This suggests great potential for modifying homozygous and complex heterozygous mutations by base editing in human embryos. In 2018, a Chinese scientist, He Jiankui, claimed to have produced the world's first genome-edited babies, twin girls, one of which was purported to have HIV resistance due to the disabling of both copies of the *CCR5* gene by CRISPR/Cas9 technique. However, this technique can also affect non-target DNA, requires multiple embryos, and is thus beset with many ethical obstacles.

If PSCs could produce oocytes or sperm, they could be useful for gene correction. There are several ways to treat genetic diseases in PSCs. Using CRISPR/Cas9 technology, DNA can be added or removed to modify mutations, after which only corrected cells would be selected for proliferation. This means that all oocytes and sperm generated by modified PSCs

would not have the disease. Unintended mutations that might occur during this process could be removed before the conversion to oocytes or sperm. Second, in the case of mosaic disease (a disease that does not affect all cells) including extra chromosomes, PSCs without this syndrome could be isolated and converted into oocytes and sperm. However, such corrective techniques are not used to prevent genetic disease because currently, standard clinical PGD is cheaper and more efficient.

PLURIPOTENT STEM CELLS (PSCS) AS A SOURCE OF GERM CELLS

In the future, we anticipate being able to generate sperm or oocytes from PSCs for oocyte- or sperm-free patients (Fig. 2). Thus far, the main source of artificial germ cells is PSCs. Below, we introduce cell types, advantages, and disadvantages of PSCs (Fig. 3).

PSCs and reproduction are closely related. The first PSC was isolated from the inner cell mass (ICM) of a blastocyst in 1981, calling to embryonic stem cells (ESC) (41). PSCs can differentiate into any type of cell in the body, not only the muscles, nerves, and skin cells, but oocytes and sperm as well, although not the placenta. Human ESCs were first established in 1998 and are used in many stem cell fields (42). During *in vitro* culture, PSCs rapidly proliferate and regenerate to produce enough cells for disease treatment, drug screening, and disease modeling (43).

Mouse ESCs express pluripotent markers such as Oct4, Sox2, Nanog, and Ssea1, and could contribute to both somatic and germ cells in the chimeric mouse. The first primate ESC was successfully isolated from the rhesus monkey in 1995 (44). Three years later, the same team reported ESCs isolated from human embryos produced by IVF for reproductive purposes. Although human ESCs represent the greatest potential for creating IVF embryos, they are allogeneic with regards to potential recipients, and their derivation and use have ethical and technical limitations (45). There are, however, alternative PSC types that could be used for

regenerative medicine: induced pluripotent stem cells (iPSCs), homozygous ESCs derived by parthenogenesis or androgenesis, and ESCs derived by somatic cell nuclear transfer (NT-SCNT).

Induced pluripotent stem cells (iPSCs)

In 2006, Japanese scientist Shinya Yamanaka showed that mouse skin cells could be made into pluripotent stem cells through reprogramming (46). Although these cells originated from somatic cells, they were called induced pluripotent stem cells (iPSCs) due to their similarities to ESCs. Yamanaka's team succeeded in transforming adult fibroblasts into iPSCs using four transcription factors: Oct4, Sox2, c-Myc, and Klf4 (46-48). These iPSCs were similar in morphology to ESCs and are capable of forming teratomas including all three germ layers. In mice, fertile adult mice were successfully generated from iPSCs (germline transmission) with tetraploid complementation.

iPSC has become an attractive alternative for autologous or allogeneic transplantation by overcoming the ethical concerns and immunogenicity of human ESCs. However, iPSCs have been reported to have several important limitations related to incomplete erasure of epigenetic markings and genetic instability. There is some evidence that they retain residual epigenetic memory, typical of parental somatic cells (49), which may lead to bias in their propensity to differentiate to different lineages (50).

Somatic cell nuclear transfer embryonic stem cells (NT-ESCs)

The somatic cell nuclear transfer (SCNT) procedure includes three major steps: enucleation, donor cell injection and fusion, and activation (51). After removing the oocyte nucleus, the

donor cell nucleus is injected and fused with enucleated oocytes. Reconstructed oocytes are artificially activated to initiate a developmental program and form blastocysts. Since its initial discovery in amphibians (52), SCNT success has produced cloned offspring across a range of mammalian species including horse, cow, sheep, goat, dog, cat, wolf, pig, rabbit, ferret, and monkey, suggesting that the reprogramming activity of enucleated oocytes is universal (51). The efficiency of cloning varied, however, depending on tissue origin, stem cell potency, and strain of nuclear donor cells (53-55).

Using SCNT protocols optimized in monkeys, human SCNT and NT-ESC derivations have been successful (56). Three main factors are necessary successful human SCNT. First, somatic cell nuclei must form spindle-like structures when caffeine is incorporated to prevent premature activation. Second, somatic cell fusion must be performed with HVJ-E, which protects the cytoplasm from premature activation. Third, the combination of electroporation and TSA improves SCNT reprogramming and blastocyst development. Serially, human SCNT was attempted using fibroblasts originated from elderly and type 1 diabetes patients (50,57). Initial gene expression and transcripts of pluripotency-related genes were similar between NT-ESCs and IVF-ESCs. (49). In addition, NT-ESCs clustered more closely with the IVF-ESCs than iPSCs (58). Based on the SCNT technique optimizations and outcomes; however, while the protocol for NT-ESC derivation has been proven, its feasibility is inhibited by the relatively limited availability and high cost of human oocytes. Clinical applications of human NT-ESCs for allogeneic use, however, are possible (45).

Uniparental ESCs

Uniparental embryos contain the entire paternal (androgenetic embryo) or maternal (parthenogenetic embryo) inherited genome. Uniparental embryos have been generated from a

variety of mammalian species including primates and humans (59). Mammalian uniparental embryos can produce ESCs but cannot produce offspring (59).

Parthenogenesis is a maternally uniparental reproduction (43). Activated oocytes with inhibited second polar body extrusion form pseudodiploid parthenogenetic embryos. Sister chromatids are separated but remain in the oocyte cytoplasm. These oocytes enter mitosis resulting in the development of diploid, partially heterozygous embryos due to crossover, and develop into blastocysts at a rate similar to fertilized embryos (60). Similar to natural activation, when the activated oocyte extracts the second polar body and induces the completion of meiosis, the parthenogenetic embryo includes a haploid genome. Embryos have thus been developed with abnormal placentation and other abnormal fetal development (61). However, parthenogenetic ESCs in mice can generate live pups through tetraploid embryo complementation, wherein recipient embryos contribute placenta only (62). These parthenogenic ESCs are similar to IVF-ESCs in terms of morphology, growth, global transcription profile, and genes involved in pluripotency (63).

Androgenetic ESCs have been established from embryos obtained by replacing the oocyte nucleus with sperm or by removing the female pronucleus from the zygote (64,65). Androgenetic ESCs showed haploid-originated homozygosity because they were derived from embryos reconstructed with a single sperm or male pronucleus (66). These ESCs also showed similar characteristics to IVF-ESCs.

Haploid ESCs

Haploid ESCs have been derived from two types of haploid embryos: a parthenogenetic embryo with oocyte nucleus, and an androgenetic embryo with the sperm nucleus. To produce

a parthenogenetic haploid embryo, a second polar body must be excluded, similar to what occurs in natural activation (67). Another method involves removing the male pronucleus from the fertilized zygote. In contrast, removal of the female pronucleus from the zygote generates an androgenetic embryo containing only a haploid paternal genome. Another approach to generating androgenetic haploid embryos is to remove the nucleus of the mature oocyte and replace it with sperm head. Since the 1970s, attempts to create haploid embryos in mice have been undertaken to understand different gene functions at early developmental stages (68). In 2011, haploid ESCs from parthenogenetic haploid embryos were generated (69), leading to the establishment of androgenetic haploid ESCs (64,65).

Androgenetic and parthenogenetic haploid ESCs show differences in gene expression due to paternal versus maternal imprints. However, both types of haploid ESCs share many characteristics with normal diploid ESCs, including gene expression pattern, *in vitro* differentiation potential, and chimeric ability after blastocyst injection (70). Injecting haploid ESC into blastocysts contributes significantly to the development of chimeric mice. During development, however, most haploid cells become diploid (70). These results indicate that uniparental diploid cells could contribute to the development of chimeric mice. Diploidization is a common observation in the differentiation of haploid ESC cultures (70). Its mechanism is largely unknown, but a point during the cleavage process wherein the haploid genome is replicated without cell division has been occurred (43).

A haploid cell is a valuable tool for genetic screening. Heterozygous mutations in diploid cells often show few or no phenotypic changes, rendering them unsuitable for studying recessive mutations (70). Mutations introduced into a haploid genome are in a hemizygous state and are phenotypically exposed; large pools of haploid cells with different mutations are thereby useful for screening (70). Haploid cells have shown homozygosity at HLA loci, a

potential source of cell therapy that could avoid immune rejection in allotransplantation (66). Haploid cells could thereby be matched to other patients with less risk of immunological rejection.

LIMITATIONS

Oocytes derived from male cells or sperm from female cells

There are several studies suggesting XY (male) primordial germ cells could naturally produce XX oocytes *in vivo* (71) and oocyte-like cell production from male multipotent cells (72,73). However, decades of studies on mammalian gamete formation and sex determination have determined that it is not possible to form oocytes from cells with a Y chromosome nor sperm from cells without Y chromosome (74). Furthermore, most of the current studies focused on genetic dysfunction in oocytes and sperm (ovaries and testes), no studies have demonstrated successful production of oocytes from XY (male) cells or sperm from XX (female) cells.

Embryo creation using sperm and oocytes from the same individual

Although it is possible to produce embryos from oocytes or sperm from a single individual, the possibility of genetically identical embryos is extremely rare. There are 23 chromosomes in oocytes and sperm made from pluripotent stem cells (PSC) that represent a random mix of chromosomes from the individual's mother and father. The fusion of a random oocyte chromosome and a random sperm chromosome produce an enormous variety of different children; the sheer number of potential combinations thereof precludes the production of genetically identical babies. Mixing chromosomes for the production of oocytes and sperm are a survival advantage, and a prohibitively large number of children would be needed for the production of a child genetically identical to one of its siblings by the same parents. Cloning takes genetic information from a single somatic donor cell (46 chromosomes) and transplants

it into an oocyte wherein the genetic material (nucleus) has been removed. These embryos are thus completely identical to one another (clones) in all cases. However, the use of cloning in human reproduction is prohibited worldwide.

Somatic cell haploidization

It has been suggested that the haploidization of the diploid somatic cell can be induced using enucleated oocytes (75). If diploid cell nuclei are transferred into immature or maturing oocytes, the cytoplasm might induce the separation of the diploid chromosome. This possibility has been studied using enucleated immature oocytes and cumulus cells as donor cells in the mouse; however, researchers concluded that the mitotic cell nucleus could not be induced to normal haploidization with chemical activation (76). Allocation of the chromatin on the meiotic spindle was abnormal, and separation of the mitotic chromosomes was similarly aberrant. In humans, somatic cell haploidization has also been tried with mature oocyte and cumulus cells, resulting in two PN zygotes produced by ICSI introducing the male parent's sperm into the reconstructed oocyte (77). However, further research in embryonic development and the possibility of establishing ESCs has yet to be conducted. At present, the potential for somatic cell haploidization remains unclear.

There are limitations to the use of somatic cells for ART. First, epigenetic memory must be contended with. Epigenetic memory is important for modulating gene functions such as genetic imprinting (78). During reprogramming, the epigenome of mature somatic cells undergoes massive rearrangements (79). A somatic cell nucleus must cease its original gene expression and re-establish the embryonic gene expression necessary for normal development. (80). However, such epigenetic rearrangements have not yet been sufficient to erase all epigenetic memory during reprogramming. In addition, egg donors are required to use somatic cell haploidization for research and clinical applications. Finally, in pre-clinical studies, a large

number of oocytes are required to study the mechanism of somatic cell haploidization, but the number of eggs available with which to do so is limited. To overcome this limitation, the generation of artificial oocytes from somatic cells such as PSCs could be an alternative for research and clinical applications that would circumvent current ethical concerns.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. Current applicable assisted reproductive technologies for mitochondrial replacement. From an MII oocyte with mutated mtDNA, spindle or PB1 is isolated and transferred to an enucleated MII oocyte with healthy mtDNA. Reconstructed oocytes by ST or PB1T are fertilized with sperm. In the zygote with mutated mtDNA, PNs or PB2 can be transferred to enucleated zygotes with healthy mtDNA. Two PNs are enucleated for PNT while only one PN is for PB2T. For SCNT, the fibroblasts carrying mutant mtDNA are transferred to enucleated MII oocytes with healthy mtDNA. Reconstructed SCNT oocytes are activated artificially. Embryos with healthy mtDNA can develop into ESCs or offspring. MII, metaphase II; mtDNA, mitochondrial DNA; ST, spindle transfer; PB1T, 1st polar body transfer; PB2T, 2nd polar body transfer; PNT, pronucleus transfer; SCNT, somatic cell nuclear transfer; ESCs, embryonic stem cells.

Figure 2. The entire cycle of germ cell differentiation and reprogramming for reproduction. PGCs are naturally produced from epiblast cells, which are generated from ICM. The PGCs migrate to the fetus and take up residence in the ovaries or testes, where they develop into oocytes or sperm, respectively. *In vitro* experiments, PSCs, iPSCs, and ESCs could be differentiated to germ cells, which could then develop into blastocysts. Blastocysts could be developed to ESCs *in vitro* or body *in vivo*. The body naturally harbors germ cells, and somatic cells in the body could be reprogrammed to derive iPSCs. PSCs, pluripotent stem cells; PGCs, primordial germ cells; ICM, inner cell mass; iPSCs, induced pluripotent stem cells; ESCs, embryonic stem cells.

Figure 3. Current available PSCs types. Regular ESCs, produced from IVF embryos, represent an ideal stem cell source for regenerative medicine; however, ethical and technical limitations regarding human use thereof remain barriers to their research and use. Uniparental ESCs include an entire maternal (parthenogenic ESCs) or paternal (androgenic ESCs) genome. To produce a parthenogenetic embryo, MII oocytes are activated artificially. Heterozygous or homozygous parthenogenetic ESCs can be generated depending on extrusion of the second polar body; extrusion for homozygous or no extrusion for heterozygous ESCs. Another method involves removing the male pronucleus from the zygote. In the generation of androgenetic embryos, the female pronucleus is removed from the zygote. Another approach is the removal of the nucleus from MII oocytes and replaces it with sperm head. These uniparental ESCs are usually generated for research, with no possibility for clinical applications currently. Fibroblasts could be reprogrammed to PSCs by SCNT or iPSC generation. The SCNT technique can cure mitochondrial disease because mtDNA mutated fibroblasts are transferred to enucleated MII oocytes with healthy mtDNA; the derived SCNT-ESCs then harbor healthy mtDNA. However, this is prevented by ethical issues surrounding oocyte use. While iPSCs are unfettered by ethical concerns, these are produced from mtDNA mutated fibroblasts directly and will still include mutated mtDNA after reprogramming. PSCs, pluripotent stem cells; ESCs, embryonic stem cells; IVF, *in vitro* fertilization; MII, metaphase II; SCNT, somatic cell nuclear transfer; iPSCs, induced pluripotent stem cells, mtDNA, mitochondrial DNA.

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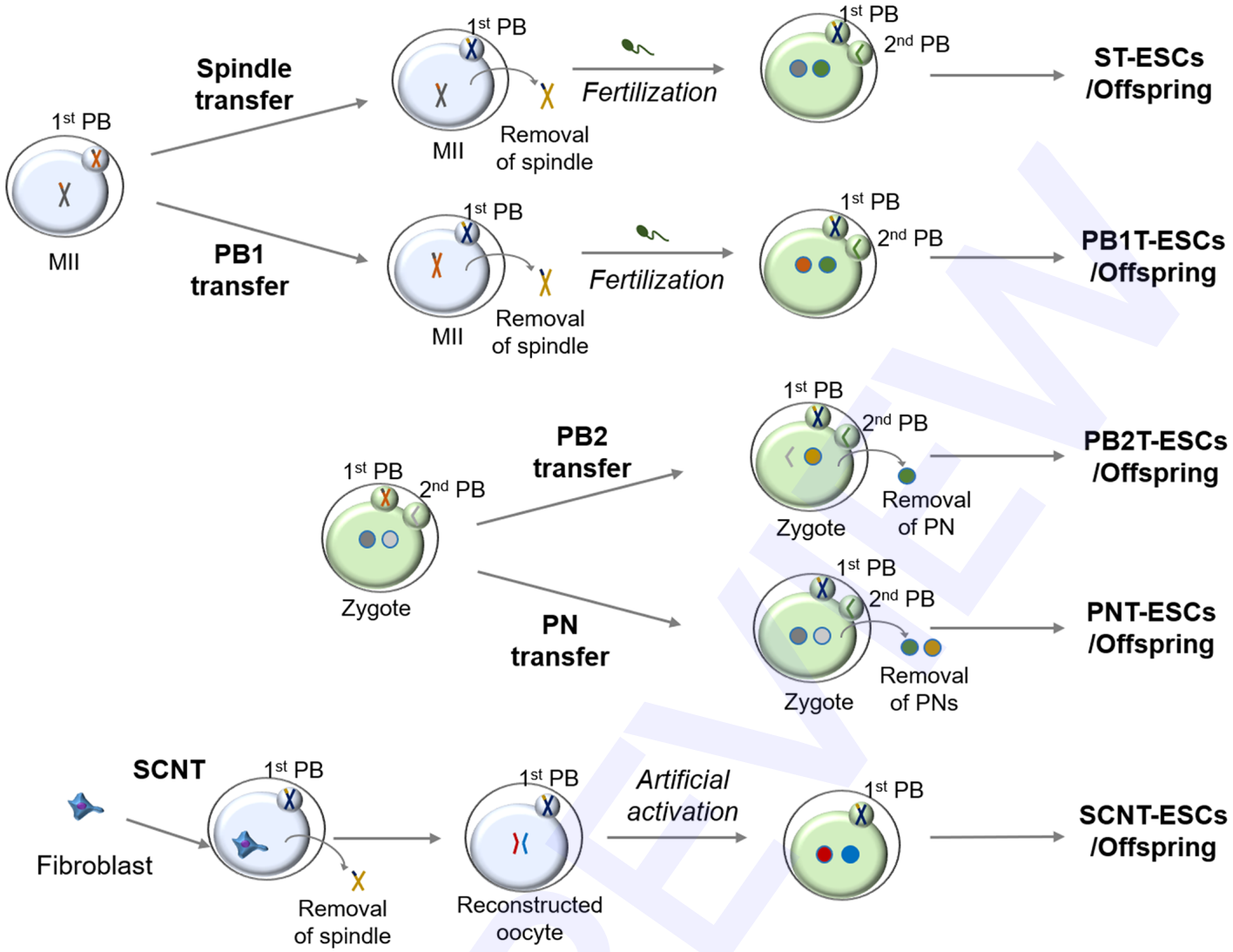

Fig. 1.

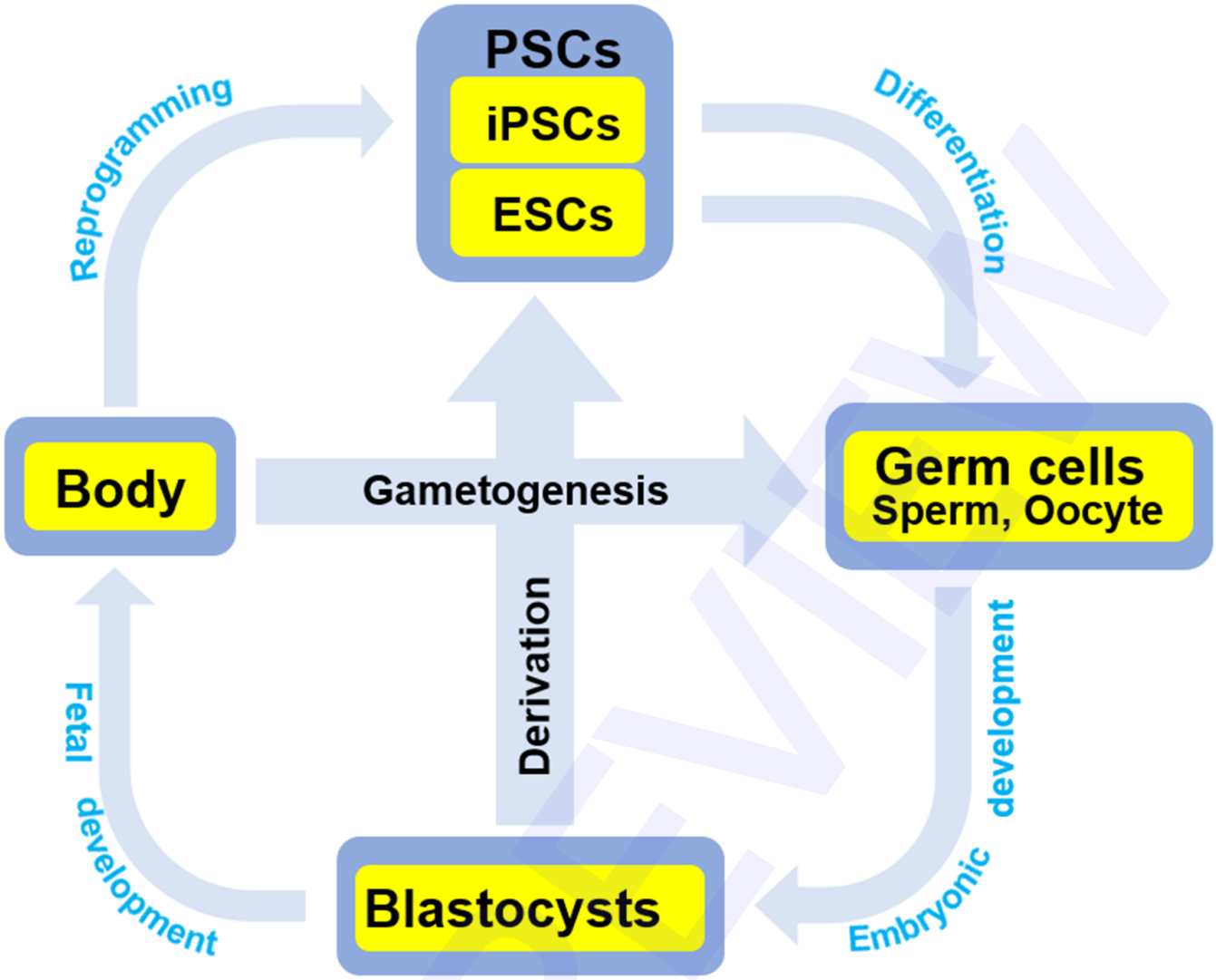

Fig. 2.

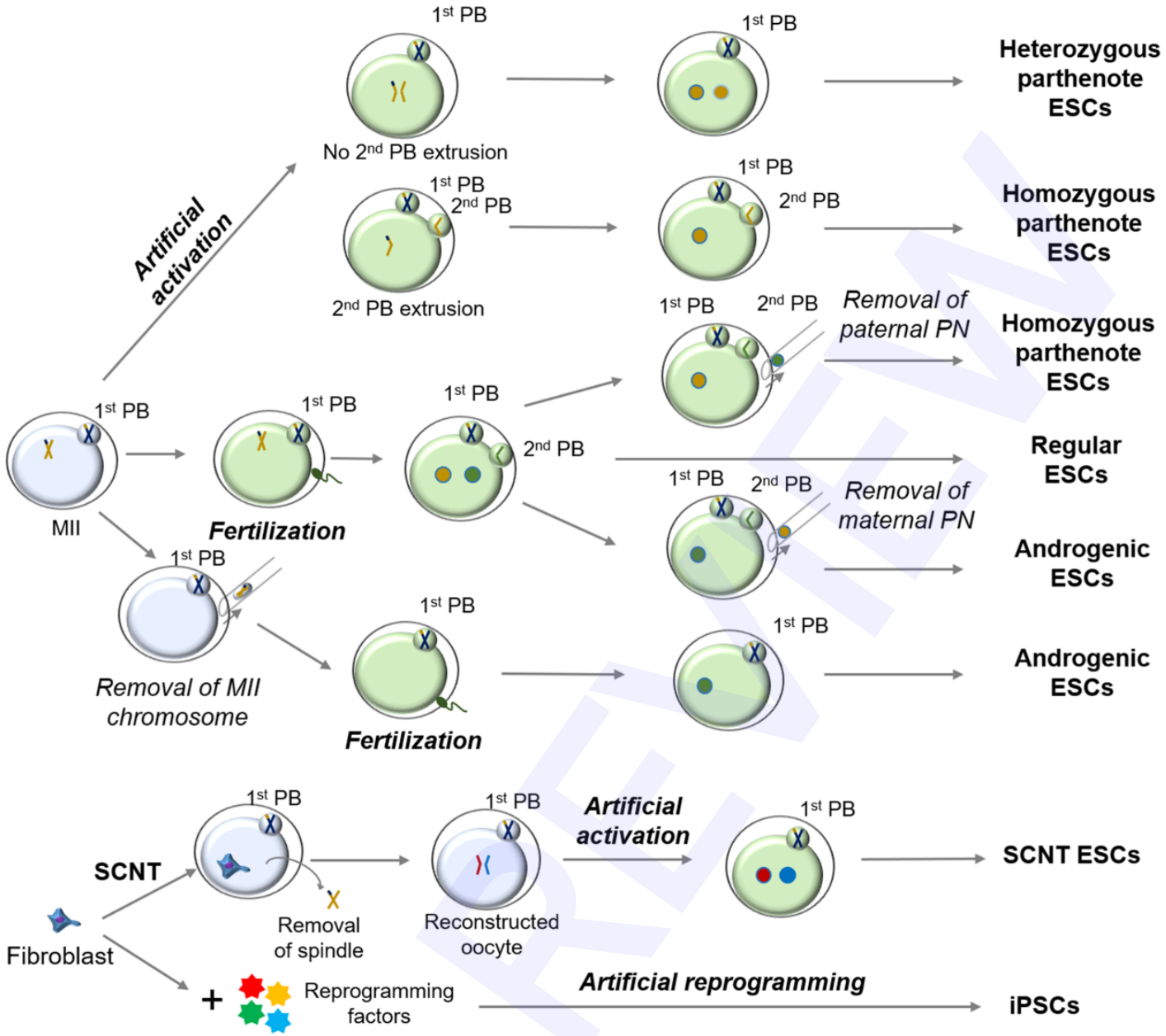

Fig. 3.