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## Adult Somatic Cells to the Rescue: Nuclear Reprogramming and the Dispensability of Gonadal Germ Cells

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### Summary

With advances in cancer therapies, survival rates in prepubescent patients have steadily increased. However a number of these surviving patients have been rendered sterile due to their rigorous oncological treatment regimens. In addition to cancer treatments, men and women, who are genetically fertile, can become infertile due to immune suppression treatments, exposure to environmental and industrial toxicants, and injury. Notwithstanding the great emotional burden from an inability to conceive a child with their partner, the financial burdens for testing and treatment are high and are rarely successful at treating these patients' sterility. Recent advances in pluripotent stem cell differentiation and the generation of patient-specific, induced pluripotent stem cells indicate that stem cell replacement therapies or *in vitro* differentiation followed by IVF may be on the horizon. Here, we discuss these recent advances, their relevance to treating male-factor and female-factor infertility, and what experimental procedures must be carried out in animal models before these exciting new treatments can be used in a clinical setting. The goal of this research is to generate functional gametes from no-greater starting material than a mere skin biopsy.

### Keywords

infertility; stem cells; assisted reproductive technology; differentiation

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## Introduction

In previous decades, medical professionals were focused on treating cancers in prepubescent boys and girls to extend their lives with little concern over preserving patients' fertility. As cancer survival rates in adolescents have steadily increased over the last few decades<sup>1-9</sup>, the focus has shifted to preserving fertility to enable these patients to produce offspring as adults<sup>3</sup>. However fertility preservation can be extended to any medical treatment that impacts fertility such as immune suppressant treatment which has been shown to cause permanent sterility in some male patients<sup>10,11</sup>. While fertility preservation will ultimately impact future patients, patients previously sterilized by their medical treatments currently have no treatment options with which to produce their own genetic offspring.

For both male and female patients experiencing infertility, the current Assisted Reproductive Technology (ART) treatment options rely solely on the premise that both partners produce functional haploid gametes. For those couples where one partner is unable to produce a functional gamete (egg or sperm), no treatment options are available<sup>12,13</sup>. Non-genetic factors that result in sterility or sub-fertility are injury, exposure to environmental and industrial toxicants, and medical interventions such as chemotherapies and immune suppressant treatments<sup>1,2,10,11,14</sup>. For these patients, there are no cures for their infertility/sterility, and they are unable to conceive a child with their partner.

For patients unable to cryopreserve gametes prior to their sterilization, two stem cell treatment options are on the horizon. 1) spermatogonial stem cell (SSC) transplant and 2) patient-specific pluripotent stem cells differentiated into functional gametes. This review will briefly discuss SSC transplant but instead focus on the use of patient-specific pluripotent stem cells as a novel means for restoring fertility in sterile patients<sup>12,13,15</sup>.

## Spermatogonial Stem Cell Transplant

The most advanced "stem cell" treatment option for patients rendered sterile by high dose chemotherapies is spermatogonial stem cells (SSC) transplant. Here, pre-pubescent boys have testis-tissue biopsies prior to gonadotoxic chemotherapy and SSCs are isolated from the samples in a GMP (good medical practice) environment and cryopreserved for re-introduction post chemotherapy. Several animal model studies have shown the ability to re-introduce spermatogonial stem cells (SSCs) obtained from testis biopsies to restore fertility in sterilized animals, <sup>16-21</sup>, including primates <sup>22</sup>. This methodology is ideal for re-introducing a patients' own natural germline stem cells for recolonization of the testes and restoration of fertility. However, this method of fertility preservation only works if a testis biopsy is obtained prior to chemotherapy. There is a large patient population that was unable to bank SSCs prior to medical interventions that caused sterility and thus are unable to be treated with this exciting new option. Likewise, this method carries a risk of re-introducing cancer cells, although this risk has been reduced as Herman et al. have shown that approximately 99% of cancer cells can be removed by flow cytometry from a testis cell suspension<sup>23</sup>. Furthermore, SSC re-colonization requires that the somatic environment of the testes must remain intact after the medical intervention. For patients with damaged somatic environments whereby the seminiferous tubules are unable to support SSC transplant due to

loss of Sertoli Cells or structural collapse of the tubules, this type of strategy is ineffective at curing a patient's infertility. In these cases, and for those patients who were unable to cryopreserve gametes or SSCs, no options are currently available to cure their infertility. However, future pluripotent stem cell options whereby functional gametes can be derived *in vitro* may be beneficial.

## Pluripotent Stem Cell Treatment Options

Recent evidence by several labs has shown the ability of human, non-human primate, and mouse pluripotent stem cells to differentiate into VASA- and DAZL-expressing primordial germ cells (PGCs)<sup>24-37</sup>, precursor cells that contribute to gametogenesis in both males and females. Studies with mouse pluripotent stem cells have even shown the ability to make functional sperm<sup>30,38</sup>. The recent work by Hayashi et. al. suggest that pluripotent stem cells can be differentiated into a PGC-like state then transplanted into a sterile mouse testis for re-colonization and the generation of functional haploid sperm cells<sup>37</sup>. While PGCs have shown the limited capacity to re-colonize sterile testis in mammals other than rodents, the possibility exists that pluripotent stem cells can be differentiated into a lineage more suitable for re-colonization and restoration of spermatogenesis. In fact, we recently demonstrated that human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) can be differentiated into SSC-like cells<sup>39</sup> that express PLZF, a marker for stem and progenitor spermatogonia. This lineage has been shown in several animal models to be capable of re-colonizing the testis as observed by SSC transplant<sup>16,22</sup>. We recently proposed a two-step concept for utilizing pluripotent stem cells to treat male infertility where sterility was caused by non-genetic factors<sup>12,13</sup>. We stated that patient-specific pluripotent stem cells could be differentiated into SSCs for transplant into the testis if the somatic environment was intact to restore fertility, or pluripotent stem cells could be differentiated into functional haploid cells for IVF if the somatic environment was unable to support germ cell re-colonization<sup>12</sup>. We demonstrated that hESCs and hiPSCs can be differentiated *in vitro* into advanced spermatogenic lineages including acrosin-, transition protein 1-, and protamine 1-positive round spermatids<sup>39</sup>. While round spermatids have not been successful in fertilizing oocytes in higher order mammals, our results indicate that it is at least feasible to differentiate pluripotent stem cells into haploid spermatids. Improvements in the differentiation strategy could lead to the maturation of round spermatids into elongated spermatids, which are capable of fertilizing an oocyte in IVF clinics or even sperm (Fig. 1). Future potential cures for infertility/sterility could target *in vitro* differentiation into functional spermatids and thus not necessitate testis cell transplantations.

Until recently, most of the major advances involving germ cell differentiation into haploid cells have been in male stem cells. The recent work by Hayashi et al. showed that mouse stem cells could be differentiated in an *in vitro/in vivo* system into oocyte-like cells that are capable of being fertilized by sperm and generating normal progeny<sup>40</sup>. Whether this outstanding achievement by Hayashi et al.<sup>40</sup> can be adapted for human stem cells remains to be seen, but this advancement is a critical step forward in generating *de novo* oocytes from human iPSCs from female patients rendered sterile by medical interventions, exposure to toxicants, or by premature ovarian failure. The major concept of this work suggested that co-culture of oocyte support cells within the follicle (granulosa cells and theca cells) can shape

the maturation of a PGC derived from pluripotent stem cells into a functional oocyte. Potentially, patient-specific pluripotent stem cells could be differentiated into follicle support cells, as shown with mouse cells<sup>41</sup>, and co-cultured with PGCs derived from the same patient-specific pluripotent stem cell line. In theory, this co-culture system could enable investigators to generate *de novo* oocytes from patient-specific pluripotent stem cells (Fig. 2)

## Sources of Patient-specific Pluripotent Stem Cells

One of the greatest advancements in the last 10 years in human pluripotent stem cell technology, as evidenced by Dr. Yamanaka being awarded the Nobel Prize, was the ability to reprogram adult somatic cells into embryonic stem-like cells using four factors: *OCT4*, *SOX2*, *KLF4*, and *c-Myc*. The resulting cells were termed induced pluripotent stem cells (iPSCs)<sup>42–48</sup>. This advance made possible the concept of generating patient-specific pluripotent stem cells in an ethically favorable method. While there have been discrepancies between embryonic stem cells (ESCs) and iPSCs in cell cycle profiles, genomic imprints, and mitochondrial function<sup>39,49–53</sup>; iPSCs appear to “get it right” upon differentiation, including establishing correct parent-of-origin imprints on at least two loci in haploid spermatids<sup>39</sup>. With the recently shown ability to generate GMP hiPSCs for potential clinical uses<sup>54,55</sup>, it may become possible to generate iPSCs from sterile patients. Advances in our protocol to GMP/animal component free conditions combined with the work of Hermann et. al.<sup>22</sup> could lead to the first stem cell replacement therapy for male infertility whereby iPSC differentiation into SSC-like cells is followed by transplantation of these SSC-like cells into the patient’s testes to restore fertility. Thus, infertility due to non-genetic root causes could be reversed using no greater starting material than a mere skin biopsy.

Similarly, the work by Hayashi et. al.<sup>40</sup> further suggest that human iPSCs could potentially be utilized to give rise to *de novo* oocytes for use in IVF clinics to allow sterile women to conceive a child, assuming fertility is solely due to a lack of functional oocytes. There is the concern that iPSCs often carry epigenetic marks similar to the original cell type and thus somewhat impact differentiation. For example, iPSCs derived from blood cells maintain epigenetic marks similar to the original blood cell type and thus differentiate into better blood cells than iPSCs derived from skin tissue<sup>56</sup>. The same problem could exist for *in vitro* derived gametes in that skin fibroblasts might not generate the most functional spermatids. Deriving iPSCs from multiple cell types and then differentiating into gametic lineages is necessary to determine which adult somatic cell type generates the most functional gamete with the most correct parent-of-origin imprints.

Until recently, iPSCs were the only source of human patient-specific stem cells. The “gold standard” of patient-specific stem cells is the generation of somatic cell nuclear transfer-derived embryonic stem cells (SCNT-ESCs). Very recently, Tachibana et. al. demonstrated the ability to generate human SCNT-ESCs from donor human oocytes<sup>57,58</sup>. The ability to use an oocyte environment to reprogram a somatic nucleus is more clinically favorable to generating iPSCs. iPSC derivations rely on the use of exogenously expressed oncogenes (*OCT4*, *SOX2*, *KLF4*, *CMYC*) to initiate cellular reprogramming into a pluripotent state. Even if these oncogenes are excised from the genome after reprogramming, there is still a

concern as to whether iPSCs possess an oncogenic potential upon transplantation. However, the ethical concerns and low efficiency surrounding SCNT-ESCs make iPSCs a far more attractive option for patient-specific stem cells. Whether SCNT-ESCs pair more closely with hESCs than iPSCs in parent-of-origin genomic imprints, cell cycle progression, mitochondrial function, among others, remains to be examined as SCNT-ESC derivations have only recently just been demonstrated in humans. Perhaps SCNT-ESCs will possess a greater ability to differentiate into more functional adult somatic and germ cells than iPSCs because SCNT-ESCs will not possess the “remnant” imprints observed in iPSCs, thereby providing a more complete reprogramming.

## Non-human Primate Animal Modeling is Essential for Progressing Stem Cell Treatments for Infertility to the Clinic

Many current animal models for infertility center on murine models. While informative, murine models for gametogenesis are distinctly different from human and non-human primate, especially with regards to spermatogenesis<sup>59</sup>. The advances by Herman et. al. show the ability of non-human primate SSCs to recolonize a sterilized testis and generate functional sperm<sup>22</sup>. To examine whether germline stem cells are dispensable and that pluripotent stem cells can be utilized to restore fertility in non-human primates sterilized by chemotherapies, SCNT-ESCs and iPSCs need to be derived from non-human primates prior to and post chemotherapy exposure (to ensure that chemotherapeutics do not negatively impact the ability to derive pluripotent stem cells capable of differentiating into germ cell lineages). SSC-like cells would need to be derived<sup>39</sup> and then transplanted into the testis to examine whether SSCs from SCNT-ESCs or iPSCs are better at recolonizing the testis, undergoing spermatogenesis, and generating the most functional haploid spermatids. Similarly, *in vitro* differentiation would need to be carried out to the haploid spermatid stage followed by ICSI (intracytoplasmic sperm(ati)d injection) into a non-human primate oocyte to determine which “patient-specific” cell source better supports oocyte activation and viable fetal development thus demonstrating whether are more capable of generating functional male gametes. Sperm derived from transplants and spermatids derived from *in vitro* differentiation will need to be assessed for correct genomic imprints on all parent-of-origin imprinted genes to ensure that resulting offspring are not in danger of inheriting a debilitating parent-of-origin imprinting disorder such as Prader-Willi. Spermatids and sperm will also need to be assessed for the ability to fertilize an oocyte, develop to the blastocyst stage, and support viable fetal development leading to live births. Likewise, long-term health and reproductive outcomes will have to be assessed.

For the female side, *in vitro* oogenesis has not been currently described in primates (see Fig. 2). However, Hayashi et. al.<sup>40</sup> pioneering work might be adaptable to non-human primates to determine whether co-culture of PGCs and follicle support cells can be matured *in vivo* by injecting in the kidney capsule or, more preferably, under the skin<sup>60,61</sup>. Oocytes derived in this manner will have to be assessed for correct parent-of-origin imprints and for functionality by IVF with donor non-human primate sperm followed by development to the blastocyst stage. All of these proposed experiments should be conducted with SCNT-ESCs and iPSCs to determine which cell source is preferable for generating functional oocytes.

Finally, for male and female gametes derived from pluripotent stem cells, the generation of a healthy progeny is the ultimate goal. Experiments absolutely must be conducted at the non-human primate level to determine whether implanted blastocysts (derived as described above for either male or female pluripotent stem cell derived gametes) can develop *in utero* correctly resulting in a healthy, normal offspring. Further examinations on post-natal development would also have to be conducted to ensure there are no cognitive and aging deficits. Addressing such questions/concerns are especially critical prior to attempting the use of pluripotent stem cell-derived gametes in the clinic to restore patients' fertility in patients rendered sterile by medical treatments, exposure to environmental or industrial toxicants, or injury.

## Conclusion

Stem cell treatments are starting to become more prevalent in clinical trials, with perhaps the first biggest advancement being the use of retinal pigment epithelial cells derived from hESCs to restore vision in patients with Macular Degeneration<sup>62</sup>. However, patient-specific cells will be required for germ cell replacement therapies at the clinical level. While the clinical applications for *in vitro* derived patient-specific gametes are still years away, the advancements being made in *in vitro* differentiation methods are making the possibility of using a patient's own somatic cells for generating functional gametes after she/he has been rendered sterile a true reality.

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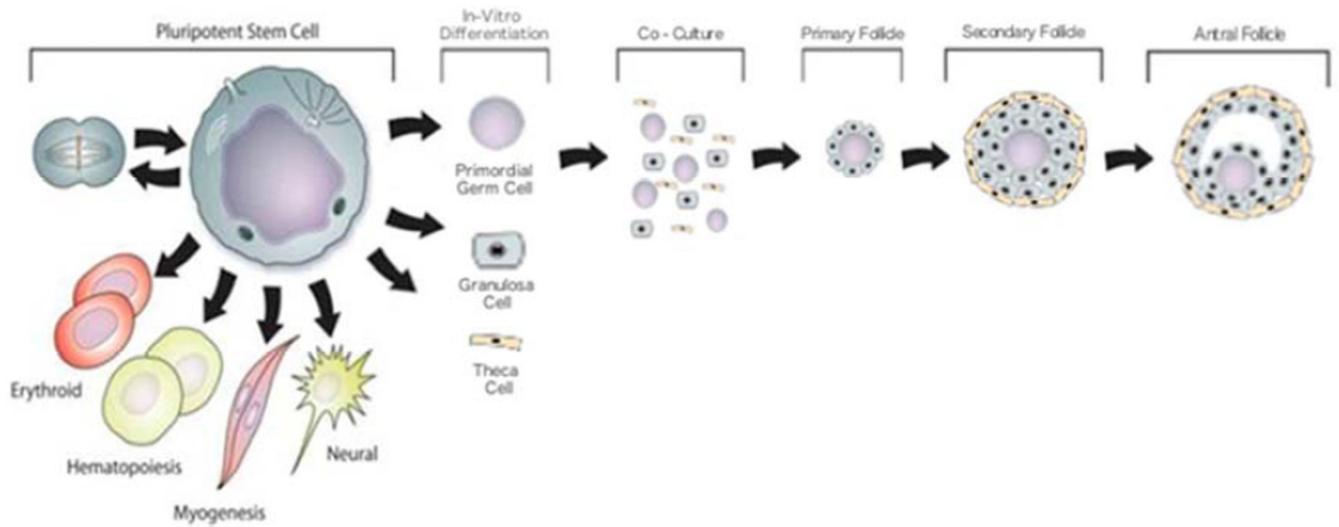
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**Figure 2. *In vitro* oogenesis**

Diagram depicting *in vitro* oogenesis whereby patient-specific pluripotent stem cells could be differentiated into primordial germ cells (PGCs) and co-cultured with follicle support cells derived from the same patient-specific pluripotent stem cells. *In vitro* maturation of the follicle would need to be performed to generate a resulting product capable of being fertilized by standard IVF methods.