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Clinical Implications of Human Spermatogenesis Initiation *in Vitro*

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With the recent publication of '*Direct Differentiation of Human Pluripotent Stem Cells into Haploid Spermatogenic Cells*' in CELL REPORTS, several fundamental and clinical avenues for future research are now available. In this article, we review the discoveries reported and also consider the implications for the management of male infertility as well as new contraceptive designs.

Key words: germ cell differentiation, haploid, spermatogenesis, spermatogonia, stem cells

INTRODUCTION

The sharp biological distinction between mortal somatic cells and potentially immortal germ cells has been held as a central tenet in developmental biology for well over a century dating back to August Weismann's Germ-Plasm Theory (for review[1]). This theory holds that whereas the germ line lineage can both maintain itself and also differentiate into somatic progeny, it is a rectified pathway in which somatic cells cannot themselves generate gametes. Cracks in this seemingly impregnable wall separating somatic and germ cells first appeared when Dolly and other cloned animals had offspring and were therefore reproductively fertile[2], since the transferred somatic cell nucleus was reprogrammed within the oocyte into a germ line lineage; explanations incorporated the idea that the oocyte's germplasm or ooplasm was vital in this process as in other systems.[3] Breakthroughs in induced pluripotency and the generation of fertile mice using tetraploid complementation embryo transfers (for review[4]) opened the floodgate by demonstrating that exposure to a just a few transcription factors could reprogram somatic cells which were rigidly committed differentiated cells into most every other cell, including cells in the germ line. Derivations of cells in the spermatogenic lineage show the promiscuity of pluripotent stem cells, and now findings of oocyte stem cells in mice capable of generating pups[5] and recently similar oocyte-like stem cells from women[6], might be another example of this cellular promiscuity *in vitro*. Whether these *in vitro* generated gamete precursors have reproductive capabilities *in vivo*, helpful for infertility patients, will be important to evaluate pre-clinically, though they will be of keen biological importance regardless.

The quest to generate viable sperm and spermatids *in vitro* from pluripotent stem cells and even somatic cells in humans and other primates has many biomedical justifications even though the endeavor is fraught with experimental and bioethical challenges.[7-9] Furthermore the stringencies which with these 'artificial sperm' are evaluated vary according the necessary endpoint. The greatest stringency is for the generation of fully functional sperm or spermatids useful and safe for reproduction in Assisted Reproductive Technology (ART) clinics. This objective is well justified by the Oncofertility Consortium, which seeks the benevolent objective of preserving fertility in male cancer survivors who were rendered infertile dur-

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ing their therapies but were also too young or fragile to produce a sperm specimen for cryobanking.[10-16] It is also justified for the treatment of infertile men suffering from either diagnosed[17] or idiopathic male infertility in cases in which neither sperm nor elongated spermatids useful for either intracytoplasmic sperm injection (ICSI) or elongated sperm injection (ELSI) can be obtained.[10-16] Discovering of the stages during spermatogenesis at which various forms of idiopathic male infertility arrest would greatly aid in the diagnoses, and perhaps eventual treatments, of these still mysterious processes. Learning of these spermatogenic arrest sites might also contribute to the design of novel contraceptives. Additionally the epigenetic modifications enabling the properly imprinted sperm chromatin and the replacement of nuclear proteins to form the sperm nucleus could be better investigated in these types of cell cultures versus in intact tissues. Anticipated improvements in the efficiency of *in vitro* spermatogenesis may also help understanding how mitochondria are modified to create the sperm mitochondria as well as how the somatic centrosome is reduced during male meiosis to form the sperm tail's basal body and the sperm centrosome.[18]

Recent studies suggest that human pluripotent stem cells (PSCs) can enter meiosis, and in some cases produce haploid products, *in vitro*.[19-21] In this review we evaluate the article just published entitled ***Direct Differentiation of Human Pluripotent Stem Cells into Haploid Spermatogenic Cells***[22], in which, we developed an *in vitro* method which achieves two significant endpoints. First, male human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) are directly differentiated into adult-type spermatogonia. Secondly, differentiating stem cells give rise to cells which are phenotypically consistent with post-meiotic round spermatids. These results highlight the full plasticity of human PSCs by showing the ability to undergo spermatogenesis *in vitro* culminating in the production of round spermatid-like, haploid cells with correct parent-of-origin genomic imprints on at least two loci. These results also contribute to the overall goal of ultimately generating gametes that may prove invaluable for understanding infertility mechanisms.

Differentiation of Human Pluripotent Stem Cells into Spermatogonia, Pre-meiotic Spermatocytes, Post-meiotic Spermatocytes and Round Spermatids

In our recently published article in *Cell Reports* entitled ***Direct Differentiation of Human Pluripotent Stem Cells into Haploid Spermatogenic Cells***, we show that culturing both human embryonic and induced pluripotent stem cells can be differentiated in mouse spermatogonial stem cell (SSC) culture conditions into spermatogonia, germline stem cells that give rise to all spermatogenic lineages culminating in the production of motile sperm.[22] Furthermore, differentiation in these conditions yields cell types consistent with pre-meiotic spermatocytes, post-meiotic spermatocytes, and round spermatids (Figure 1).

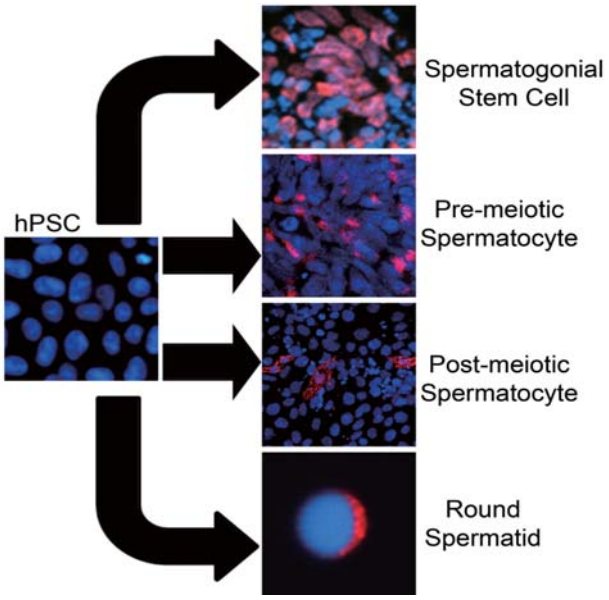

Fig. 1 **Human Pluripotent Stem Cells Can Differentiate into Spermatogonial Stem Cells, Pre-meiotic Spermatocytes, Post-meiotic Spermatocytes, and Round Spermatids**. Human PSCs do not express germ cell markers (left), but upon differentiation in mouse SSC conditions, advanced spermatogenic lineages appear, including PLZF-positive spermatogonial stem cells (top), HILI-positive pre-meiotic spermatocytes (top middle), HIWI-positive post-meiotic spermatocytes (bottom middle), and acrosin-positive round spermatids (bottom). DNA labeled with Hoechst, red staining patterns are indicative of PLZF, HILI, HIWI, and acrosin.

During *in vivo* germ cell specification, genomic imprints are removed at the primordial germ cell stage and then re-established during spermatogenesis.[23] In mice, differentiating PSCs into functional germ cells results in progeny that exhibit epigenetic disease phenotypes.[24,25] One explanation was improper imprinting during gametogenesis.[26] Haploid spermatid products produced by our protocol show correct parent-of-origin, genomic imprints

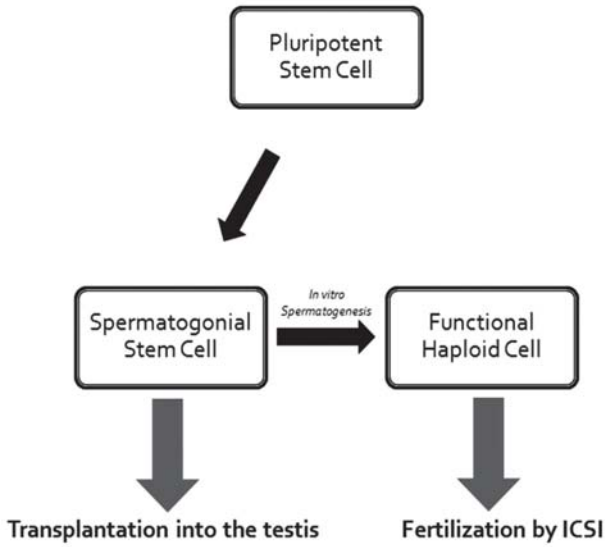

Fig. 2 **Human PSCs Can Give Rise to Cell Types Useful in Clinical Restoration of Fertility**. Diagram depicting how our differentiation strategy yields SSCs, which can be useful for restoring fertility by transplantation into the testis, and haploid spermatids which can potentially be used to fertilize an oocyte by IVF.

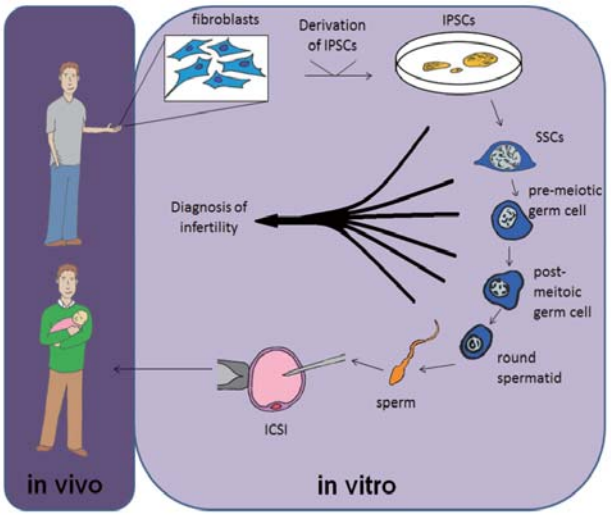

Fig. 3 **Patient-specific Stem Cells Can Be Used for Diagnosing and/or Treating Infertility**. Diagram showing how adult somatic cells can undergo *in vitro* spermatogenesis and be used to diagnose and treat infertility.

on at least two loci.[22] While we have not determined whether an individual cell progresses all the way from a diploid pluripotent stem cell to a haploid spermatid, we do show that major cell types observed during spermatogenesis are obtained from our differentiation protocol. Thus this differentiation protocol could be highly useful in diagnosing and developing treatments for idiopathic infertility.

Using In Vitro Spermatogenesis to Diagnose and Treat Infertility

Infertility affects perhaps 15% of couples worldwide, with male factors responsible for 40-60% of all cases.[27] In men without a genetic root cause for infertility, stem cell transplantation represents a possible treatment option to restore fertility.[28-31] Clinical interventions such as chemotherapy and immune suppressant treatments often render male patients sterile. Protocols to preserve future fertility in boys undergoing cancer therapies who cannot yet bank their own sperm are under development.[11,32-37] However for adult and prepubescent patients rendered sterile prior to sperm collection, there are no current treatments to restore fertility.

Our differentiation protocol generated two endpoints critical for driving in vitro spermatogenesis to the clinic to treat infertility in patients without a known genetic etiology. First, human PSCs were differentiated into SSC-like cells, cells that reside at the foundation of spermatogenesis. Several previous studies have shown the ability of human and non-human primates PSCs to differentiate into primordial germ cells (PGCs).[19,21,38-43] Although this cell lineage has the capability of restoring fertility in rodents, including PGCs derived from mouse PSCs[44,45], SSCs remain the gold standard for colonizing cells which recapitulate spermatogenesis following transplantation.[46,47] Thus differentiating PSCs into SSCs is an important step in the future ability for using patient-specific PSCs to restore fertility, as SSCs derived from PSCs can be transplanted into the sterilized testes to restore spermatogenesis (Figure 2). Furthermore, the sperm generated following transplant would, in theory, be the patient's own genetic material.

However, transplantation of SSCs derived from PSCs supposes that the somatic environment of the testis remains intact. Prolonged clinical interventions, injury, exposure to environmental toxins, etc. can cause sterility and render the somatic environment useless for SSC transplantation. For these patients, complete spermatogenesis *in vitro* is critical for generating haploid products useful for ART procedures to fertilize a partner's oocyte and pass along their own genetic material (Figure 2). Our differentiation protocol generates haploid products

consistent with round spermatids. While techniques for utilizing round spermatids to fertilize oocytes have not been proven in human and non-human primates, our differentiation protocol at least shows the feasibility of generating haploid products that could be useful in *in vitro* fertilization (IVF). This would suggest that functional haploid cells may be obtained from no greater starting material than a skin biopsy needed for iPSC derivations (Figure 3).

In vitro spermatogenesis also holds great promise to diagnose male infertility and provides a novel tool for exploring root causes for male infertility. By deriving hiPSCs from infertile men, such as from patients with Sertoli-cell-only (SCO) syndrome, followed by direct differentiation with our protocol, we can examine where spermatogenesis arrests, and in the case of SCO patients, identify whether hiPSCs can differentiate into SSCs and whether viability of SSCs is a major concern. A similar strategy can be implored for men with defects in Leydig Cell function, DAZ-family deletions and even Klinefelter Syndrome. In cases where spermatogenesis arrests *in vitro*, chemical screens can be employed with a read-out for haploid cell production to identify novel compounds that could treat known causes for male infertility. In this same light, chemical screens can be utilized to discover male forms of birth control that temporarily arrest spermatogenesis but do not endanger SSC survival. Thus the clinical uses for in vitro spermatogenesis are substantial and could lead to the first cures for male sterility.

Ethical Considerations for In Vitro Spermatogenesis

As briefly mentioned above, the ethical concerns for utilizing *in vitro* spermatogenesis in a clinical setting should be considered. The benevolent goal of restoring fertility in a sterile male is noteworthy, but only if the result allows the patient to pass along his own genetic material to his offspring. There are studies that suggest that hiPSCs are not identical to their parent cell lines due to the reprogramming process's strain on the epigenetic makeup of the parent line[48-50], notwithstanding the inability right now to efficiently generate clinical grade iPSCs. If these results hold true, then truly patient-specific stem cells would be unattainable with current methodologies and would render *in vitro* spermatogenesis useless as an infertility treatment. However, *in vitro* spermatogenesis would still be useful for chemical screens, identification of novel root causes for infertility, pathways critical in spermatogenesis, among others.

Related to whether iPSCs are truly patient specific is the concept 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.[51] The same problem could exist for *in vitro* spermatogenesis in that skin fibroblasts might not generate the most functional spermatids. Deriving iPSCs from multiple cell types and then differentiating in our protocol is necessary to determine which adult somatic cell type generates the most functional sperm cell lineage.

Another ethical concern would be the imprinting status of the haploid products generating by *in vitro* spermatogenesis. To date, we have shown that haploid products derived by our protocol are epigenetically similar to fertile human sperm on two loci[22], but all imprinted genes would have to be examined before this technique could be utilized in a clinical setting. Human imprinting disorders exist, and recent reports suggest that IVF babies show a slight increase in incidences of rare imprinting disorders.[52] Whether IVF with spermatids derived from adult somatic cells would show a higher incidence in imprinting disorders would need to be investigated.

CONCLUSION

While the risks and ethical considerations for moving *in vitro* spermatogenesis to the clinic are great, the potential rewards are sufficient to continue to explore this option to treat male infertility. To date, our methodology needs to be refined to use xeno-free conditions to generate haploid spermatids for use in the clinic. As advances in *in vitro* spermatogenesis are made, this technique may become fundamental in diagnosing and treating a currently incurable disorder: male infertility.

DISCLOSURE

All authors declare that this study has no conflict of interest.

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