

# In Vitro–Derived Gametes from Stem Cells

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## Abstract

Sperm and eggs are essential cells for reproduction and fertility in mammals. Lack of sperm production is one of the leading causes of infertility, a major and growing problem in the developed world affecting 13 to 18% of reproductive-age couples. The birth of the first test tube baby by in vitro fertilization marked an advance in infertility treatment. Later on, several important new techniques called assisted reproductive technologies were developed to help couples who experience infertility. One limiting factor is the requirement of reproductive cells (gametes) for use in in vitro fertilization. For azoospermic men lacking sperm cells, producing gametes in vitro could be a new window to overcome infertility. In the past few years, several reports have been published on generating germ cells from stem cells, one of the epitomes of which was the report on functional in vitro–derived (IVD) germ cells. These mature haploid sperm cells from mouse embryonic stem cells were capable of egg fertilization and producing live offspring. In tandem with previous advancements in germ cell research, development of new technologies based on IVD gametes will change the future of infertility and provide a new basis for the establishment of novel therapeutic approaches to cure more complicated conditions of infertility. In addition, IVD gametogenesis provides an accessible system for studying the specification and differentiation of sperm cells and related processes such as meiosis, morphogenesis, and motility.

## Keywords

- ▶ infertility
- ▶ stem cells
- ▶ germ cells
- ▶ IVD gametes

Infertility is a major and growing problem in the developed world affecting 13 to 18% of reproductive-age couples.<sup>1</sup> A series of conditions may lead to infertility including decreased sperm counts, increasing age of first-time parents, increase in the incidence of polycystic ovary syndrome, and the rising incidence of sexually transmitted diseases.<sup>2</sup> The

birth of the first test tube baby by in vitro fertilization in 1978 marked an advance in infertility treatment. Later on, several important new techniques, collectively called human assisted reproductive technologies (ART), were developed to help couples who experience infertility.<sup>3</sup> However, some patients devoid of germ cells such as azoospermia patients cannot

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benefit from ART treatments. Several approaches have been developed to obtain an alternative source of normally developed spermatozoa and oocytes including in vitro-derived (IVD) gametes for infertile couples. Recently two main techniques for producing gametes in vitro, somatic cell haploidization (nuclear transfer, cloning) and germ cell differentiation from embryonic stem cells (ESCs), were successfully developed in mammals, opening a potential new window for the treatment of human sterility.<sup>3</sup> During haploidization, somatic cells (2N) go through induced meiosis, and the diploid chromosomes are reduced to a haploid (1N) state to form gametes.<sup>4</sup> Haploidization can be achieved by introducing a diploid somatic cell into cytoplasm that is preprogrammed to undergo meiosis. These haploid (1N) cells can then be potentially utilized as an infertility treatment.<sup>3,4</sup>

There is an increasing interest by scientists and clinicians in applying stem cell technologies in the reproductive field, in particular generating gametes in vitro.<sup>5-30</sup> Much of the interest has centered on the creation of functional gametes from stem cells to study the complicated processes that occur during germ cell development and applying this technology in clinical treatment. New research offers promising developments in establishing novel stem cell-based approaches for the generation of germ cells in various stages of differentiation from mice and human stem cells, from both embryonic and nonembryonic cells.

It has been shown that ESCs are able to differentiate spontaneously and rapidly into germ cells of various stages following induction utilizing a myriad of culture systems. It appears that IVD germ cell development occurs in a similar manner as their in vivo derived counterparts.<sup>5-8</sup> More surprisingly, scientists could report live offspring following the injection of IVD sperm-like cells into oocytes.<sup>8</sup> This demonstrated the possibility of deriving functional and mature gametes from ESCs. The exact mechanism by which gamete-like cells are generated during stem cell culture is still unclear.

There is some evidence that germ cells can be obtained from adult stem cells residing in bone marrow, peripheral blood cells, and fetal skin cells. This could indicate that adult tissue-derived stem cells may provide a new source for in vitro produced germ cells. However, derivation of fully differentiated gametes from non-ESCs has not yet been reported. In addition, recent studies have shown that stem cells in bone marrow and peripheral blood may serve as a reservoir for new gametes in mouse ovaries and that reprogramming may not be required.<sup>9,10</sup> Having a pool of stem cells capable of giving rise to germ cells in organs like bone marrow or peripheral blood may one day make it possible to delay the age of normal menopause and preserve fertility in female cancer patients where chemotherapy often leads to gamete damage.<sup>10</sup>

In this review after a short introduction on germ cell biology and development, we discuss in vitro derivation of germ cells in various stages, from primordial germ cells to fully developed gametes, from embryonic and nonembryonic stem cells, and the differences in generating male and female gametes.

## In Vitro-Derived Germ Cells from ESCs

Hübner et al published the first report of IVD germ cells in 2003.<sup>5</sup> They demonstrated that mouse ESCs (mESCs), both male and female, in culture can differentiate into female germ cells precursors that can begin meiosis and then form follicle-like structures. In vitro oocyte-like cell derivation without specific changes to culturing condition suggested spontaneous differentiation of germ cells from ESCs. In this study, mESCs were transfected with a vector harboring a modified version of oct4 promoter to drive a germ cell-specific expression of green fluorescent protein (GFP).<sup>5</sup>

Shortly after the first in vitro demonstration of ESC differentiated into oocytes, the first attempt to produce male germ cells was performed by Toyooka et al.<sup>6</sup> mESCs were aggregated into EBs, which are three-dimensional structures that induce differentiation and mimic the early embryo environment producing cell types of the three germ layers (ectoderm, endoderm, and mesoderm). They also obtained mouse vasa homolog (Mvh), a robust germ cell-specific marker, positive cells from mESCs through embryoid body (EB) formation. Using a bone morphogenetic protein (BMP) 4 producing M15 cell line as feeder cells resulted in enhanced germ cell formation within EBs. To track and isolate germ cells in culture, the Mvh gene was tagged with GFP. GFP-positive cells were sorted by fluorescent-activated cell sorting (FACS) and co-cultured with gonadal cells, followed by transplantation beneath the testis capsule of male mice to examine further in vivo development of newly formed germ cells. Implanted cells showed engraftment into the lumens of seminiferous tubule-like structures distinct from those of the host testis. Some of these cells gave rise to morphologically normal spermatozoa. However, functional data concerning the fertilization capacity of IVD male germ cells upon injection into donor oocytes was still needed to determine the full potential of these cells.<sup>6</sup>

Geijsen et al also developed a system capable of inducing spontaneous differentiation of primordial germ cells (PGCs) from mouse ESCs through EB formation.<sup>16</sup> Adding retinoic acid (RA), a growth factor produced in the testis that promotes PGC proliferation, enhanced mESCs differentiation into SSEA-1+ cells, which were then magnetic-activated cell sorted based on the SSEA-1 markers. The authors showed that IVD germ cells displayed erasure of the methylation pattern in some imprinted loci, a hallmark of normal germ cell development.<sup>6</sup> These cells were further differentiated into postmeiotic germ cells and formed haploid cells. Intracytoplasmic injection of sorted cells into oocytes led to the biological activation of eggs and the formation of blastocysts in 20% of the cases of fertilized oocytes. It was not tested if the embryos were capable of developing normally with uterine transfer.<sup>16</sup>

Taking a step forward, Clark et al showed for the first time that human ESCs (hESCs) were capable of differentiating into early germ cells.<sup>7</sup> These investigators utilized EB differentiation of female and male hESCs to produce mixed-cell populations spontaneously. A subset of these differentiated cells expressed markers belonging to different stages of germline

cells such as Stella, DAZL, and c-Kit. Furthermore, the expression levels of the primordial germ cells marker mouse vasa homolog (Mvh) and meiotic marker SYCP3 were increased in EBs. Collectively, the results of this study showed hESCs of both sexes spontaneously form putative germ cells and undergo advanced stages of germ cell development entering into meiosis. Interestingly, it was shown that germline-associated genes such as DAZL were expressed in undifferentiated hESCs. This suggested that a subpopulation of hESCs may not be truly undifferentiated and may contain some cells committed to the germ cell lineage.<sup>7</sup>

Efforts to derive female germ cells continued with a study by Lacham-Kaplan et al in 2006 in which ESC-derived EBs were cultured in conditioned medium obtained from testicular cell cultures of newborn male mice.<sup>11</sup> This unique culturing system led to the generation of oocyte-like cells enclosed within follicular-like structures with one or two layers of flattened cells that expressed oocyte-specific markers such as ZP3. Further characterization of these cells is required to determine the detailed morphological structure of the zona pellucida. It is also necessary to determine the specific stage of derived germ cells and further examine their functionality.<sup>11</sup>

Novak et al explored the ability of male mESCs to differentiate into germ cells.<sup>12</sup> They report the successful derivation of follicle-like structures similar to those seen by Hübner et al.<sup>5,12</sup> Although the expression of meiotic proteins SYCP3, SYCP1, SYCP2, and Rec8 were reported, surprisingly, formed germ cells were not able to continue through meiosis. These IVD cells showed no signs of synapsis, recombination, or appropriate segregation of chromosomes, which are all key elements of early meiosis.<sup>12</sup>

Qing et al explored a novel two-step approach to derive female germ cells from mESCs.<sup>13</sup> First they obtained PGCs through EB formation followed by a second step where EBs were co-cultured with a fetal ovarian granulosa layer. Derivation of PGCs was detected by analysis of PGC-specific markers. An increase in gene expression of the meiotic marker SYCP3 and oocyte-specific markers such as GDF9 were reported in differentiated cells. The GDF9-positive cells resembled an immature oocyte without zona pellucida formation.<sup>13</sup>

Chen et al also reported the development of a ovarian follicle-like structure from hESCs through EB formation.<sup>14</sup> Detection of c-KIT and low amounts of Mvh was observed. However, it seems that three-dimensional EB formation is not essential for oocyte-like formation.<sup>14</sup>

Kerkis et al reported the derivation of both male and female germ cells from mESCs through EB formation with the addition of RA.<sup>15</sup> Depending on the length of culture time, they showed that male germ cells appeared in the medium. Then the production of sperm-like cells could induce female germ cell generation. Interestingly, IVD sperm could penetrate and fertilize an IVD oocyte, leading to structures resembling blastocysts.<sup>15</sup>

Another study using mESCs transfected with a reporter gene (GFP) associated with GDF9 gene promoter, a female germ cell marker, through EB and monolayer differentiation

was performed by Salvador et al.<sup>16</sup> Cells rapidly became GFP+ and displayed an oocyte phenotype.

The first report of functional sperm derived in vitro was shown by Nayernia et al using a two-step sorting method after monolayer differentiation of mESCs.<sup>8</sup> They reported the production of live offspring from IVD sperm. PGCs were obtained from mESCs with a Stra8-driven GFP reporter after germ cell induction with RA. These cells were then isolated by FACS. Sorted cells were then grown in RA-supplemented medium and transfected with a second reporter, Prm1-DsRed. Using a two fluorescent reporter system enabled them to track the progression of mESC-derived germ cells through the spermatogenic process and isolate cells with a sperm-like morphology. The injection of sorted sperm-like cells resulted in blastocysts and pregnancies after transferring into uterus. Most of the fertilized eggs appeared to die, yet a few offspring were born. However, due to developmental complications presumably because of the mESC to IVD germ cell differentiation process, mouse pups died prematurely. This result suggests that epigenetic reprogramming of the derived sperm-like cells may not have been complete.

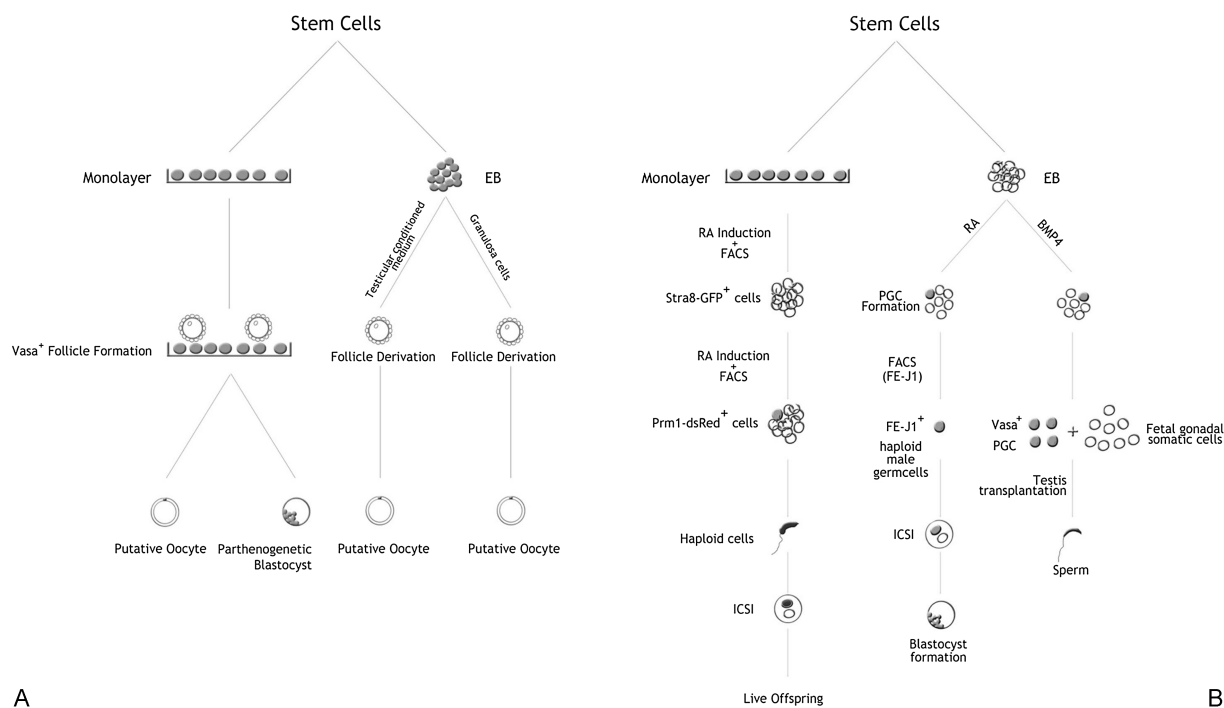
## Generation of Germ Cells from Non-ESCs

Several reports have demonstrated that adult stem cells are able to differentiate into early germ cells. In mouse, an elevated expression of Mvh has been observed in bone marrow-derived mesenchymal stem cells treated with BMP4.<sup>25</sup> However, differentiation of bone marrow cells to germ cells is restricted to early stages of germ cell development with additional factors likely being required to obtain more mature cells.<sup>26</sup> Similar germ cell differentiation potential has been shown in human bone marrow-derived mesenchymal stem cells.<sup>27</sup> In addition to germ cells, Lue et al observed differentiation of bone marrow-derived mesenchymal stem cells to testicular somatic cell lineages,<sup>28</sup> which raises novel possibilities for the treatment of male infertility in human by stem cell-based therapeutic approaches.<sup>27,29</sup> Generation of germ cells and gametes from embryonic stem cells is summarized in ►Fig. 1.

## Outlook

### Improvement of IVD Germ Cell Technology

One of the major concerns in the clinical application of IVD germ cells is the potential of those cells to produce healthy progeny. Mice produced by sperm cells derived from ESCs showed health problems due to inappropriate reestablishment of paternal imprinting genes during gametogenesis and embryogenesis.<sup>8</sup> However, this problem potentially has been overcome with a recent study using multipotent adult germ cells (maGSCs) derived from testis to produce IVD sperm cells.<sup>30</sup> The differences in the epigenetic state of gametes derived from ESCs and maGSCs may be due to the starting populations, with ESCs carrying somatic imprinting versus the germ cell imprinting of maGSCs. In the normal physiologic state of an early mouse embryo, PGCs are formed in response to BMP signaling and activation of the germ cell gene



**Figure 1** Schematic representation of in vitro-derived (IVD) (A) female and (B) male germ cells and gametes from embryonic stem cells. EB, embryoid body; FACS, fluorescent-activated cell sorting; ICSI, intracytoplasmic sperm injection; PGC, primordial germ cell; RA, retinoic acid.

expression.<sup>31</sup> These cells, still having somatic imprinting, migrate in the embryo through the dorsal mesentery and hindgut where they undergo imprinting erasure shortly before arriving at the genital ridge. This erasure of somatic-type imprinting is important in PGCs because these cells require germ cell specific imprinting to pass on to the next developing embryo. In male mice, reinstatement of imprinting starts in pro-spermatogonia at embryonic day 13.5 (E13.5). In addition, these cells undergo retrotransposon repression to safeguarding genome integrity by virtue of a subset of DNA- and histone-modifying enzymes and piwi family proteins. In marked contrast with male germ cells undergoing genome imprinting well before meiotic activity, the female germ cells start this epigenetic process during meiosis around prophase I.<sup>31</sup> In contrast with their male counterparts, female germ cells do not require the activity of the genome-safeguarding proteins because their mitotic proliferation is very limited relative to the massive proliferation activity of spermatogonial germ cells after puberty.<sup>31</sup> Therefore the normal molecular events of imprinting need to be accurately recapitulated in any in vitro technique aiming at generating IVD gametes to culminate in the birth of healthy offspring.

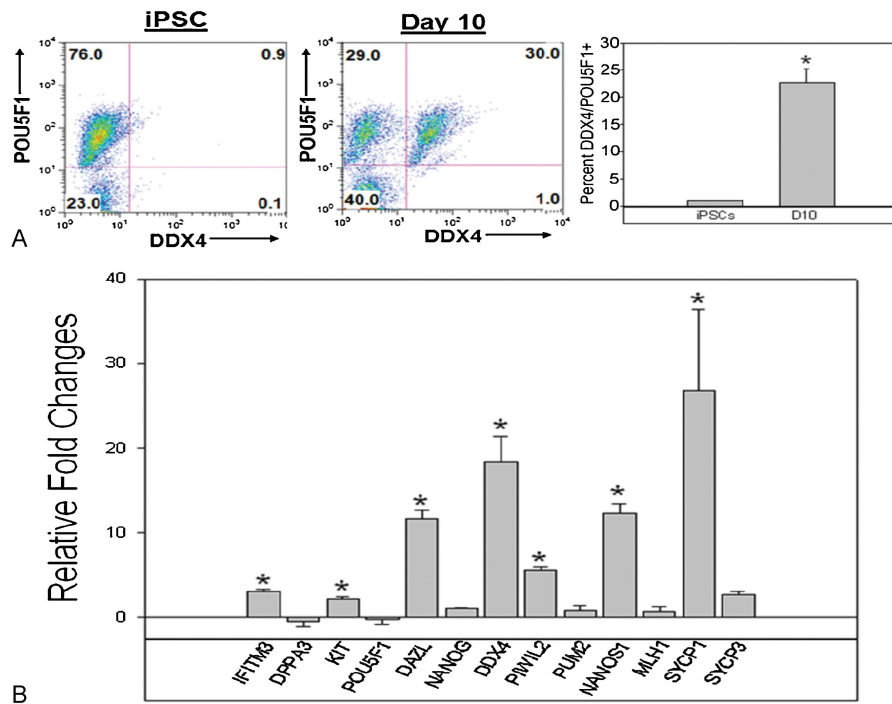
Other problems in clinical applications of IVD gametes are the immunologic and genetic challenges of stem cell therapy. Application of IVD gametes derived from autologous stem cells provides a solution to overcome this problem. Mesenchymal stem cells and induced pluripotent stem (iPS) cells are promising sources for the derivation of gametes. Previously, direct differentiation of skin cells to germ cells was shown.<sup>32–34</sup> We demonstrated clearly that

iPS cells derived from skin fibroblasts are able to differentiate to early stages of germ cells and express specific markers for primordial germ cells (→Fig. 2). Derivation of germ cells from iPS cells of infertile patients and comparing them with those from normal individuals also offers an excellent system to study pathologic germ cell specification and differentiation. This would be a major advance in developing a model to elucidate the causes of infertility and other reproductive disorders that are initiated during embryonic development.

### IVD Sperm as a Model for Nanorobots

Another important aspect of IVD sperm is its application as a mechanical model in developing medical nanorobots. The nanomechanical systems for medical applications (e.g., drug delivery system for the treatment of cancer patients) will significantly expand the effectiveness, precision, and speed of future medical treatments while at the same time significantly reducing their risk, cost, and invasiveness. Sperm-like derived cells exhibit the formation of flagella. Flagella comprise the biological apparatus necessary for movement and penetration of the egg and therefore can be used as means to drive the nanorobots.

Many aspects of flagellar motion have been studied, but none of these studies have determined the exact equation for the flagella abnormalities. Most of them are restricted to linear theory or weakly nonlinear theories<sup>35</sup> and are based on small deformation assumptions. Because the deformation of flagellum in nano-scale is very large with respect to its own size, the small deformation assumption does not hold. To study the dynamics, stability, and deformation of flagella, a



**Figure 2** Stem cells derived from skin differentiated to germ cells. Human-induced pluripotent stem cells (hiPSCs) were differentiated for 10 days on mouse embryonic fibroblast feeder cells in 20% KSR media. This system relies on spontaneous germ cell signaling in culture as a result of not passaging and every other day media changes as previously done in our laboratory.<sup>22–24</sup> (A) Flow cytometry revealed a significant (\*statistically significant relative to iPSCs;  $p < 0.05$ ) increase in the number of DDX4/POU5F1+ (22.75%) oocyte-like cells (OLCs) postdifferentiation when compared with undifferentiated counterparts (0.95%). Day 10 differentiation cultures also demonstrated significant (\*statistically significant relative to iPSCs;  $p < 0.05$ ) upregulation of the germ cell premigratory genes *IFITM3* and *KIT*, migratory genes *DAZL* and *DDX4*, the postmigratory genes *PIWIL2* and *NANOS1*, and the meiotic gene *SYCP1*.

geometrically exact nonlinear model with initial twist and curvature must be considered. Sperm whose locomotion is empowered by propelling are tiny examples analogous to large-scale vehicles that are used and studied in aerospace. Rotorcrafts such as helicopters develop the required force for locomotion in a way analogous to the way a sperm does—but in a different direction. Multidisciplinary studies combining biology, mechanics, and electronics may help us to design an appropriate model for developing biological apparatuses for developing nanorobots.

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