

Review Article

Stem Cells as New Agents for the Treatment of Infertility: Current and Future Perspectives and Challenges

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Stem cells are undifferentiated cells that are present in the embryonic, fetal, and adult stages of life and give rise to differentiated cells that make up the building blocks of tissue and organs. Due to their unlimited source and high differentiation potential, stem cells are considered as potentially new therapeutic agents for the treatment of infertility. Stem cells could be stimulated *in vitro* to develop various numbers of specialized cells including male and female gametes suggesting their potential use in reproductive medicine. During past few years a considerable progress in the derivation of male germ cells from pluripotent stem cells has been made. In addition, stem cell-based strategies for ovarian regeneration and oocyte production have been proposed as future clinical therapies for treating infertility in women. In this review, we summarized current knowledge and present future perspectives and challenges regarding the use of stem cells in reproductive medicine.

1. Introduction

Nearly 72.4 million people or 15% of couples experience fertility problems [1]. For couples and clinicians, a diagnosis of infertility signals the start of investigations and possible treatment. Infertility, defined as failure to conceive a clinically detectable pregnancy after >12 months of unprotected intercourse, is a common condition, reported by 1 in 6 couples [1, 2]. As infertility is a heterogeneous condition, caused by various underlying pathologies, it is possible that some of the mechanisms leading to infertility also play a role in the etiology of this outcome [3–5]. In recent years, several advancements have been made in assisted reproduction treatment and now more than 80% of couples experiencing infertility issues can conceive a child [6].

Due to their unlimited source and high differentiation potential, stem cells are considered as potentially new therapeutic agents for the treatment of infertility. In this review,

we will summarize current knowledge regarding the use of stem cells in reproductive medicine.

2. Stem Cells: A Novel Hope in Cell-Based Therapy

Stem cells are undifferentiated cells that are present in the embryonic, fetal, and adult stages of life and give rise to differentiated cells that are building blocks of tissue and organs (Table 1). In the postnatal and adult stages of life, tissue-specific stem cells are found in differentiated organs and are instrumental in repair following injury to the organ. The major characteristics of stem cells are (a) self-renewal (the ability to extensively proliferate), (b) clonality (usually arising from a single cell), and (c) potency (the ability to differentiate into different cell types) [7, 8]. Totipotent or omnipotent cells are the most undifferentiated cells and

TABLE 1: Characteristics of stem cells used in stem cell-based therapy of infertility.

ESCs	MSCs	Stem cell from extraembryonic tissues	iPSCs	Spermatogonial stem cells
Derived from inner cell mass of the blastocyst	Derived from bone marrow, adipose tissues, bone, Wharton's jelly, umbilical cord blood, and peripheral blood	Derived from amnion, chorion, placenta, and umbilical cord	Derived from somatic cells	Derived from testicular tissues
Pluripotent	Multipotent	Multipotent	Pluripotent	Pluripotent
These cells can differentiate into cell types of all three germ layers	These cells can differentiate into mesoderm-derived tissues (adipose tissues, bone, cartilage, and muscle)	These cells can differentiate into adipocytes, endothelial cells, hepatocytes, osteocytes, myocytes, and neurons	These cells can differentiate into cell types of all three germ layers	These cells can differentiate into cell types of all three germ layers
Prolonged proliferation	Degree of proliferation depends on the tissue from which these cells were isolated	Degree of proliferation depends on the tissue from which these cells were isolated	Prolonged proliferation	Difficult to be maintained in cultures
Indefinite self-renewal potential	Limited self-renewal	Limited self-renewal	Indefinite self-renewal potential	Self-renewal ability to go through numerous cell divisions while maintaining the undifferentiated state
High telomerase activity	Low telomerase activity	Low telomerase activity	High telomerase activity	High telomerase activity
Immortal; cell lines remain intact for long periods of time and produce endless numbers of cells	Production of limited number of cells	Production of limited number of cells	Immortal; cell lines remain intact for long periods of time and produce endless numbers of cells	—
These cells are not immune privileged	These cells have immunomodulatory characteristics	—	These cells are not immune privileged	These cells are not immune privileged

are found in early development. A fertilized oocyte and the cells of the first two divisions are totipotent cells, as they differentiate into both embryonic and extraembryonic tissues, thereby forming the embryo and the placenta [9]. Pluripotent stem cells are able to differentiate into cells that arise from the 3 germ layers—ectoderm, endoderm, and mesoderm—from which all tissues and organs develop [10]. Commonly, stem cells are derived from two main sources: early embryos (embryonic stem cells (ESCs)) and adult tissue (adult stem cells).

ESCs are pluripotent stem cells derived from the inner cell mass of the blastocyst [11]. The essential characteristics of ESCs include derivation from the preimplantation embryo, prolonged proliferation in their pluripotent state, and stable developmental potential to form derivatives of all three embryonic germ layers [11].

Mesenchymal stem cells (MSCs) are one of the most common adult, multipotent stem cells [12]. They can be derived from a variety of tissues including bone marrow, adipose tissue, bone, Wharton's jelly, umbilical cord blood, and peripheral blood [13]. MSCs are adherent to cell culture dishes and are characterized by specific surface cell markers. MSCs show variable levels of expression of several molecules, CD105 (SH2), CD73 (SH3/4), stromal antigen 1, CD44, CD166

(vascular cell adhesion molecule), CD54/CD102 (intracellular adhesion molecule), and CD49 (very late antigen), and lack the expression of surface markers characteristic for HSCs (CD14, CD34, CD45, and CD11a/LFA-1), erythrocytes (glycophorin A), and platelet and endothelial cell (CD31). MSCs are able to differentiate into mesoderm-derived tissue such as adipose tissue, bone, cartilage, and muscle [13–16]. Recently, MSCs were differentiated into neuronal tissue which is derived from the ectoderm. This is an example of transdifferentiation, that is, when a cell from one germ layer (mesoderm) differentiates into neuronal tissue (ectoderm) [17].

Stem cells can also be derived from extraembryonic tissues (amnion, chorion, placenta, and umbilical cord) [18]. Amnion and chorion contain stromal cells that display characteristics and differentiation potential similar to bone marrow-derived MSCs and are able to differentiate into adipocytes, endothelial cells, hepatocytes, osteocytes, myocytes, and neurons [7, 18]. Placental-derived stem cells have the capacity to differentiate into ectodermal, mesodermal, and endodermal cell types, while umbilical cord matrix stem cells, after transplantation, enhanced muscle regeneration in mouse model of severe muscle damage and promoted blood vessel formation and neurological function

TABLE 2: Potential advantages and disadvantages of stem cells in regenerative medicine.

Stem cells	Advantages	Disadvantages
ESCs	Pluripotent; high telomerase activity	Ethical concerns; malignant potential; difficult to control; may require many steps to differentiate into desired cell type; immune rejection
MSCs	No ethical or moral concerns; low malignant potential; avoiding allogeneic immune rejection	Limited flexibility; multipotent; difficulty to be maintained in cell culture for long periods
Stem cell from extraembryonic tissues	No ethical or moral concerns; reducing risk of tumorigenicity	Limited flexibility; multipotent
iPSCs	No ethical or moral concerns; patient-specific cells	Use of viral vectors to introduce genes; malignant potential
Spermatogonial stem cells	No ethical or moral concerns	Relatively small numbers in testis; difficulty to be maintained in cultures; immune rejection

in animal models of ischaemic brain disease [18]. The main advantage of stem cells derived from extraembryonic tissues is the efficient isolation from tissues normally discarded at birth avoiding ethical concerns that plague the isolation of human embryonic stem cells [7] (Table 2).

Recently, Takahashi and Yamanaka [19] generated pluripotent cells by reprogramming somatic cells. These cells are called induced pluripotent stem cells (iPSCs) and share similar characteristics with ESCs: exhibiting morphology of ESCs, expressing ESCs markers, having normal karyotype, expressing telomerase activity, and maintaining the developmental potential to differentiate into derivatives of all three primary germ layers. Thus, iPSCs are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells [20]. Transplantation of stem cells or their derivatives into respective tissues or organs is considered as one of the most promising remedies for many incurable diseases. Unfortunately, immune compatible cells are hardly obtainable for any given patient because of the specificity and complexity of human immune system. In this regard, induced pluripotent stem cells (iPSCs) and gene editing technologies are believed to offer an unprecedented solution for obtaining sufficient healthy autologous cells [21]. However, it should be emphasized that, despite numerous technical advances in the reprogramming technology, iPSCs apart from a very small number of ongoing clinical studies are not yet ready for transplanting into patients. Relatively little is known about iPSCs molecular and functional equivalence to hESCs and careful analysis of the genomic and epigenomic integrity of human iPSCs is required before their therapeutic use.

Stem cells could be stimulated *in vitro* to develop various numbers of specialized cells including male and female gametes suggesting their potential use in reproductive medicine.

3. Stem Cells Are Novel and Unlimited Source for Male Gametes: True or False?

During past few years a considerable progress in the derivation of male germ cells from pluripotent stem cells has been

made [22–24]. These studies provide a desirable experimental model for elucidating underlying molecular mechanism of male germ cell development and potential strategies for producing haploid germ cells for the treatment of male infertility.

Spermatogenesis is a complex process by which spermatogonial stem cells (SSC) self-renew and differentiate into haploid spermatozoa. In mammals, this process takes place in the seminiferous tubules of testis, which provide a functional niche for male germ cells [25] and involve three major stages: mitosis, meiosis, and spermiogenesis [24]. Errors at any stage of spermatogenesis can result in subfertility and infertility [26].

SSC reside in adult testis and maintain spermatogenesis and continual sperm production throughout a male's lifespan [27]. SSC are diploid cells that originate from less differentiated primordial germ cells that migrate to the gonadal ridges during embryogenesis [28]. SSC can be found in the seminiferous tubule, lying near to the basement membrane [29]. Several markers could be used for the identification and isolation of SSC: spermatogonia-specific marker Stra8 for mouse SSC [30, 31], thymocyte antigen 1 (Thy-1), CD9, stage-specific embryonic antigen-4 (SSEA4), $\beta 1$ and $\alpha 6$ integrins for rat SSC [32], SSEA4, and G-protein coupled receptor 125 (GPR125) for human SSC [33]. SSC are a potential tool for the treatment of male infertility due to their ability to differentiate into male gametes *in vitro* and capacity to restore male fertility *in vivo* [34, 35]. SSC are adult stem cells, but SSC-derived cells, called multipotent adult germline stem cells (maGSC), have differentiation potential similar to ESCs. *In vitro*, maGSC are able to spontaneously differentiate into derivatives of all embryonic germ layers and are able to generate teratomas after transplantation in immunodeficient mice [31]. Nolte and coworkers showed that maGSC are able to undergo meiosis and form haploid male germ cells *in vitro* [30]. An important breakthrough for SSC-mediated spermatogenesis was made by Hermann and coworkers [35]. They showed that autologous and allogeneic SSC transplantations into the testes of adult and prepubertal recipient macaques, which were rendered infertile with alkylating chemotherapy, regenerate spermatogenesis resulting in production of functional sperm. These results strongly indicate SSC transplantation as a novel and successful therapeutic tool for male infertility caused

by chemotherapy before puberty [35]. Although SSC seem to be a good candidate for the stem cell-based therapy of male infertility, a low concentration of SSCs in mammal testis and challenges associated with protocols for their isolation, identification, and culturing have to be addressed before their clinical use [29].

Hübner et al. first reported the successful derivation of gametes from mouse embryonic stem cells (ESCs) *in vitro* [36]. Afterwards, different studies with mouse ESCs have shown the ability to make functional spermatozoa [37, 38] capable of giving rise to live offspring after use of intracytoplasmic injection [37]. Differentiation of male germ cells from human ESC has also been demonstrated [39–43]. Similarly, studies with human ESCs have revealed the ability to differentiate *in vitro* into advanced spermatogenic stages, including round spermatids which are not capable of fertilizing oocytes in high-order mammals [22, 23].

Besides the fact that ESCs are genetically unrelated to the patient in need of fertilization treatment, the isolation of human ESCs is ethically controversial because it involves the destruction of human embryos. The significant breakthrough in stem cell biology, a discovery of patient-specific induced pluripotent stem cells (iPSCs), may overcome these issues. Recently, several studies have reported that both mouse iPSCs [24, 44, 45] and human iPSCs can differentiate into male germ cells [20, 21].

It has been verified that mouse iPSCs can form functional spermatozoa [46, 47]. Functional assays have shown that spermatozoa generated from iPSCs were capable of fertilizing the oocytes after intracytoplasmic injection and giving rise to fertile offspring following embryo transfer [46]. So far, functional male gametes from human iPSCs have not been obtained.

There are two possible approaches in generating of male germ cells from pluripotent stem cells: *in vitro* differentiation into advanced, haploid cell products [20, 21] or combined *in vitro* differentiation and *in vivo* transplantation [24, 48]. Generally, there are two methods to produce male gametes from the pluripotent stem cells *in vitro* [20]: the monolayer differentiation and the embryoid body (EB) formation [24, 44]. Direct differentiation on monolayers of human fibroblast ensures more consistent differentiation results compared with EB formation [20].

Different growth factors or cytokines could induce pluripotent stem cells into germ cells *in vitro* (Figure 1), such as bone morphogenetic protein 4, stem cell factor, epidermal growth factor, and forskolin, but most of *in vitro* differentiation protocols include retinoic acid (RA) induction [46, 49, 50]. It has been shown that RA, an active derivative of vitamin A, regulates the timing of meiotic initiation in mice [50, 51]. Some protocols include combination of RA and testosterone [45] or subsequent exposure to differentiation cocktail containing forskolin, human leukemia inhibiting factor (LIF), bFGF, and CYP26 inhibitor R115866 [22]. Testosterone is required for spermatogenesis *in vivo* and stimulates Sertoli cells to produce different growth factors, including stem cell factor that promotes germ cell differentiation [52]. Forskolin is involved in meiosis induction [53] and induces germ cell proliferation by activation of cyclic

adenosine monophosphate [54]. LIF promotes survival and proliferation of gonocytes [55], while bFGF helps balancing self-renewal and differentiation of SSC [56]. R115866 acts by suppressing the inhibitory effects of CYP26 on STRA8, the meiosis regulator gene [51].

In some studies combination of *in vitro* differentiation followed by *in vivo* transplantation was performed in order to gain male gametes in advanced differentiation stages [24, 48] (Figure 1). Most often, pluripotent stem cells are induced in SSC-like stages *in vitro* and then transplanted into sterile mice testis [24] or ectopic, into the dorsal region of the mice, together with immature testicular cell suspension [44, 48]. It has been shown in several animal models that SSC-like cells are capable of recolonizing the testis [34, 35] and exhibit proper spermatogenesis [46]. A limiting step for stem cell replacement therapy of infertility could be the damaged somatic environment of the testis. If the somatic environment is damaged it is not receptive to SSC transplantation and thereby not able to restore patient fertility [57]. Ectopic cotransplantation of SSC with testicular cells might be a way to overcome this limitation but Yang et al. have reported that, although iPSC-derived germ cells could reconstitute seminiferous tubules and settle at basement membrane, no further differentiation was observed in reconstituted seminiferous tubules [44].

4. Stem Cell-Derived Oocytes: Current Knowledge and Future Perspectives

Stem cell-based strategies for ovarian regeneration and oocyte production have been proposed as future clinical therapies for treating infertility in women.

There has been a long-persisting dilemma regarding the presence of ovarian stem cells in adult mammalian ovaries. Several research studies claimed that they have identified functional oogonial stem cells in the postnatal ovary of several different species including humans and now there is steadily increasing experimental evidence on their existence [58]. An important breakthrough was made by Zou and his coworkers who successfully established long-persisting pluripotent/multipotent ovarian stem cell lines in neonatal and adult mice [59]. They detected cells residing within the ovarian surface epithelium of neonatal and adult mice that were double positive for mouse vasa homologue (MVH) and DNA marker 5-bromodeoxyuridine (BrdU) confirming that these cells were of germ cell lineage and exhibited a replicative potential (Figure 2). With passage in culture, the cells isolated by Zou et al. were confirmed to have significant proliferative capacity and expressed high telomerase activity, *Oct4*, and *Nanog*. The cells were then marked using a retroviral vector bearing green fluorescent protein (GFP) before being directly delivered into the ovaries of adult female mice rendered sterile by treatment with chemotherapy. Importantly, GFP⁺ follicles in various stages of maturation were observed several weeks later in the ovaries of the conditioned mice indicating that isolated ovarian stem cells were capable of regenerating functional oocytes when transplanted back into sterile recipient mice [59] (Figure 2).

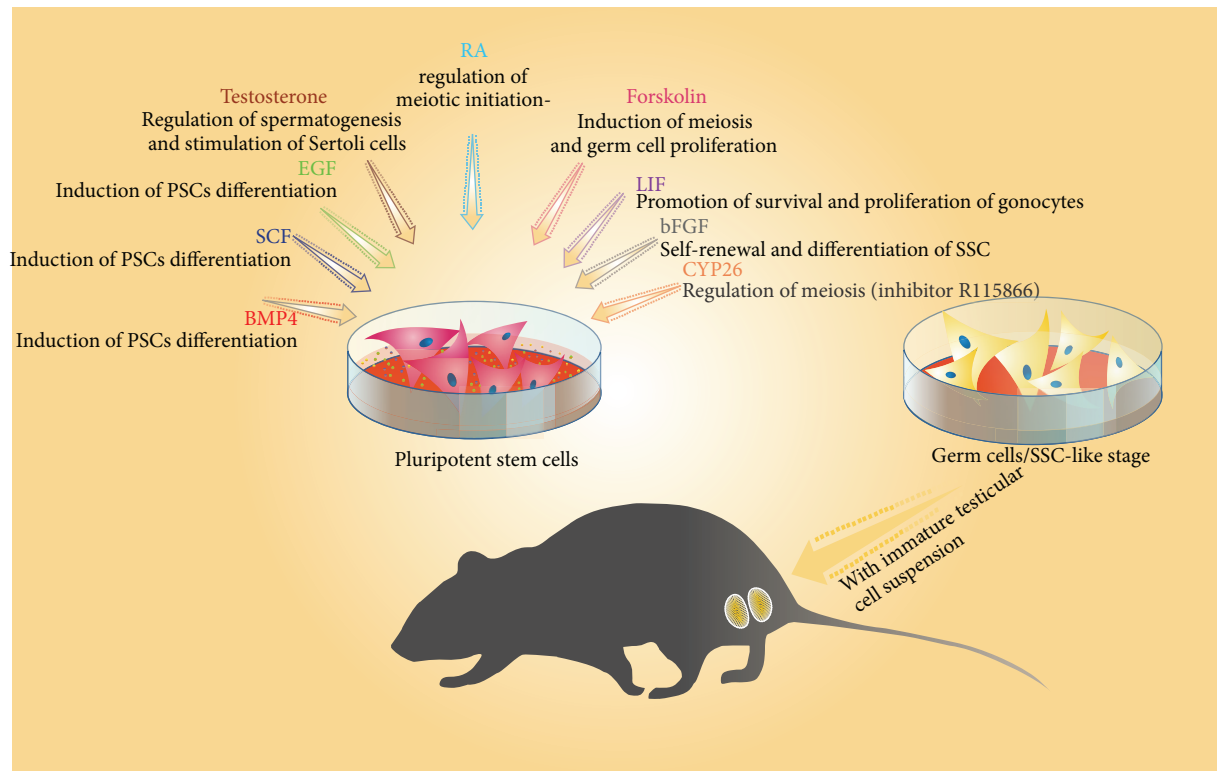


FIGURE 1: Stem cell-derived male gametes. Several growth factors and cytokines are used for *in vitro* differentiation of pluripotent cells into male gametes/SSC-like cells. The transplantation of stem cell-derived SSC-like cells in sterile mice results in proper spermatogenesis.

Recently, the work by White et al. has identified a rare population of mitotically active germ cells in human ovaries that can be purified and cultured *in vitro* to spontaneously form oocytes [60]. These cells, named as germ stem cells (GSCs), were isolated from reproductive-aged human ovaries using fluorescence-activated cell sorting (FACS) with an antibody against the carboxyl (-COOH) terminus of the germ cell-specific marker Ddx4, which is expressed on the cell surface of GSCs. Further, GSCs were capable of forming oocyte-like structures and incorporating into follicles under specific *in vitro* and *in vivo* conditions. This work highlights a unique potential to generate oocytes *in vitro* from isolated cells in reproductive-aged women who may have a depleted follicle pool from such genetic defects as fragile X-associated primary ovarian insufficiency. This recent advance, along with those described above, highlights the unique methodologies being developed to combat female-factor infertility representing a significant step towards the revolutionary idea of neo-oogenesis in reproductive-aged women through the isolation and characterization of germ stem cells.

However, despite the discovery of the potential germ stem cells in mammalian ovaries, it remains uncertain whether these cells exist and function in ovaries under physiological conditions. Liu et al. concluded that active meiosis, neo-oogenesis, and GSCs are unlikely to exist in normal, adult human ovaries [61]. Findings published by Zhang et al. contradict the results obtained by White et al. and Zhang et al. used fluorescent proteins to identify GSCs in the ovaries

of mice, but these cells failed to divide or differentiate into oocytes [62]. The scientific community has questioned both the methods and significance of these studies. Supporters of postnatal de novo oogenesis disagree with the study conducted by Zhang et al. and state that the study investigated oocytes and not GSCs in their applied experimental setting; thus, the researchers never observed mitosis in Ddx4-positive cells since oocytes expressing cytoplasmic Ddx4 do not divide [63]. According to White et al., Ddx4 is found on the cell surface of GSCs and thus enables FACS-based isolation of living GSCs from adult mouse and human ovaries [60]. This is in contrast to the opinion of Zhang et al., who argue that Ddx4 is expressed only in the cytoplasm and not on the cell surface and hence FACS-based isolation of GSCs is problematic [64]. While the debate continues, only future experiments will help to clarify this issue.

In the meantime, several studies were published regarding the potential of pluripotent stem cells for differentiation into oocytes. Eguizabal and coworkers managed to generate haploid female cells from human pluripotent stem cells, but neither of them resembled an oocyte nor is predicated to possess a functional ooplasm capable of being fertilized [22]. However, 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 spermatozoa and generating normal progeny [65]. This outstanding advancement further shows the ability of pluripotent stem cells to differentiate into all cells of the

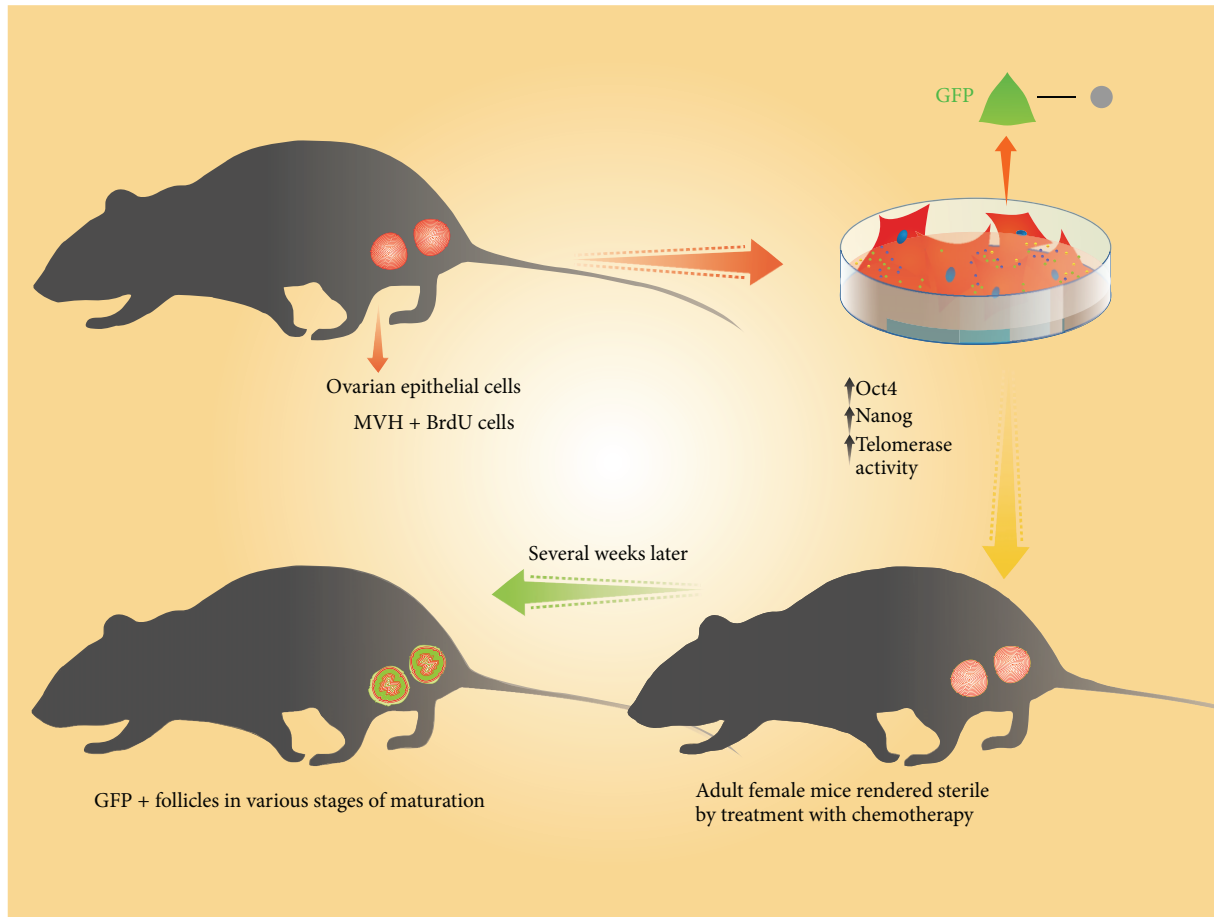


FIGURE 2: Ovarian stem cells: isolation and regenerative potential. Ovarian stem cells (MVH+BrdU+ cells) residing within the ovarian surface epithelium of neonatal and adult mice express high telomerase activity, Oct4, and Nanog and have a capacity to generate functional oocytes when transplanted back into sterile recipient mice.

adult organism [64]. Whether the work by Hayashi and colleagues can be adapted for human stem cells remains to be seen, but this advancement is a critical step forward in generating functional de novo oocytes from human iPSCs obtained from female patients rendered sterile by medical interventions, exposure to toxicants, or premature ovarian failure [64].

5. Conclusions

Pluripotent stem cells open new perspectives in the treatment of patients with azoospermia. Although the use of ESCs is connected with many ethical concerns, there are no ethical issues regarding the use of iPSCs. Moreover, ESCs are genetically unrelated to the patients, while it may be possible to get offspring with their own genetics by using iPSCs in derivation of functional male gametes.

The potential clinical applications of putative ovarian derived stem cells are apparent. The development of techniques to prolong the window of fertility for women has the ability to meet the needs of future populations and their delay in childbearing. If a viable source of oocyte production remains in infertile women with a reduced ovarian follicle

pool, for example, due to chemotherapy or advanced age, the potential exists to restore fertility in these women. The identification of GSCs gives hope to these women and suggests the potential for fertility restoration. In future, the protocols for isolation and culture of GSCs must be optimized. In the meantime, production of germ cells from ESCs or iPSCs is another possible alternative for the treatment of infertility.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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