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# In Vitro Spermatogenesis; Past, Present, and Future

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## Abstract

The study of culturing spermatogonial stem cells (SSCs) dates back to the 1950s. However, regeneration of complete spermatogenesis process *in vitro* is still a greater challenge. Studying spermatogenesis *in vitro* is significant in elucidating germ cell biology, and the knowledge may be useful for genetic manipulations of defective germ cells or producing transgenic animals, fertility preservation, and treatment of infertility. Fertility preservation would be more beneficial for adult and prepubescent patients who develop sterility due to gonadotoxins. Discovering of the stepwise stages in spermatogenesis and various forms of arrests at specific stages would help in the diagnosis of especially, idiopathic infertility and deciding treatment options. Different techniques have been tried to differentiate stem cells into germ cells over decades. A larger number of studies has used genetically manipulated stem cells to achieve differentiated germ cells. In contrast, differentiation of stem cells directly into SSCs bypassing the step into primordial germ cells (PGCs) to minimize time frame and employing techniques involved in least genetic manipulations are other important techniques to increase utilization within a clinical setting. As the use of transfected cell lines disqualifies the putative gametes obtained for clinical applications, trying to generate patient-specific germ cell with least genetic manipulations will be more effective in future applications, especially for patients with pre-pubertal cancer and azoospermic men who desire to become biological fathers.

**Keywords:** spermatogonial stem cells, germ cells, 3D culture, sperm, infertility

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## 1. Introduction

Treatment of male infertility is always a challenge. Understanding correct pathological processes is difficult and time consuming as different etiologies may be responsible for a given semen parameter abnormality. It has been estimated that 8% of men in reproductive ages seek

medical reproductive assistance [1]. Among half of subfertile couples, male factor is the causative or contributory factor and according to our experience around 12% of subfertile men are severe oligozoospermics or azoospermics. In majority of azoospermia cases, especially in non-obstructive azoospermics, the etiologies are genetically manipulated, and treatment with foci of sperm using assisted reproductive technologies (ART) may carry a greater chance of genetic diseases in the outcome. *In vitro* matured sperm are a greater hope for those men to attain the biological fatherhood.

Spermatozoa are not only act as a vehicle for delivering paternal DNA to the oocyte, but also robustly contribute to epigenetic processes in embryogenesis. Sperm DNA and the chromatin structure as a unit, drive genes toward activation or silencing upon delivery to the egg [1]. For better performance, it has to undergo several rounds of morphological, biochemical, physiological, and epigenetic changes during spermatogenesis. Even after leaving the testis, sperm is subjected to further maturation in the epididymis, and is not fully competent to do the deterministic task until mixing with accessory gland secretions and activating several proteins, cytokines and signaling pathways. All those processes are interrelated with the stability of DNA it carries.

Modeling spermatogenesis will ease the study of complex biological interactions *in vitro*, in relation to gametogenesis and fertilization (i.e., epigenetic processes, transcription, translation variations, activation or silence of signaling pathways, etc.). In addition, the modeling will helps to elaborate genetic disorders or other pathological conditions and to discover new drugs (fertility drugs or contraceptive drugs). Rapid development of the field of bioinformatics is immensely useful toward this progress. However, the use of artificial gametes for fertility treatment is far from the vicinity considering the questions still to be answered.

One of the technical challenges in the study of spermatogenesis is lack of a proper *in vitro* model to recapitulate the process [2]. Recent development of techniques and technologies such as different cell culture systems gene cloning/transgenic animals, gene expression, gene silencing, mass spectrometry and microarray, etc., has immensely been contributed to identify a plethora of endogenous and exogenous factors in the regulation of this process. Compiling all these data in a proper order, for example, grouping expressed genes, proteins, and metabolites into functional categories may allow in recapitulating spermatogenesis process and better understanding of the underlying mechanisms of normal and abnormal pathways [3].

Attempts to make gametes outside the body (*in vitro*) or outside its niche (*ex vivo*) have been ongoing for more than a century [4], and there is a rapid escalation of research in the past decade. Main approaches in achieving this goal include (a) autologous or allogenic testicular transplantation of SSCs, stem cells or differentiated putative germ cells from other sources; (b) auto or xeno-grafting of testicular tissues, SSCs, differentiated putative germ cells into other parts of the body, for example, under the skin; (c) *in vitro* culture of SSCs, stem cells or differentiated putative germ cells with testicular tissues/cells (organ culture systems) or without testicular tissues; (d) sperm cloning, etc. Two main barriers encountered in the *in vitro* spermatogenesis process are; haploidization of stem cells or progression beyond pachytene stage, and inability to further differentiation of the few round spermatids obtained by culture, especially up to formation of tail.

## 2. Understanding the natural process of spermatogenesis

### 2.1 Models to describe spermatogenesis

Spermatogonial stem cells represent a very rare population of germ cells consisting about 0.03% (20,000–35,000) in adult mouse testes [5] or even lesser 2000–3000 [6]. Self-renewal of SSCs and spermatogenesis are described using different models. Among them, more detailed studies have been done with mouse. The “A-single” ( $A_s$ ) model originally proposed by Huckins et al., and according to them two types of SSCs are present in the seminiferous tubules; Type A and Type B. Type A—SSCs are more primitive due to absence of heterochromatin, while Type B cells are more differentiated as their nuclear heterochromatin content is high. Type A spermatogonia are subdivided into three groups according to their topological arrangements in the seminiferous tubule; A-single ( $A_s$ ), A-paired ( $A_{pr}$ ), and A-aligned ( $A_{al}$ ). Division of  $A_s$  spermatogonia leads either to produce individual two new cells (self-renewing cells) or connected two cells by intercytoplasmic bridges ( $A_{pr}$ ). Further divisions of  $A_{pr}$  lead to formation of  $A_{al}$  or chains of 4, 8, 16, and occasionally 32 cells.  $A_s$  represents the stem cell pool and same characteristics may remain among few  $A_{pr}$  cells as well. Larger chains of  $A_{al}$  (8, 16, 32) differentiate toward the Type A1 spermatogonia and then give rise to A2, A3, A4, Intermediate and B, respectively. These differentiated spermatogonia divide in a synchronize manner and found at specific stages of the seminiferous epithelial cycle. B spermatogonia differentiate into spermatocytes, and they undergo further divisions by meiosis to produce secondary spermatocytes and haploid spermatids, respectively. Single  $A_{pr}$  cell passes eight mitotic steps resulting 1024 spermatocytes, and total 4096 haploid spermatids from subsequent meiotic division. Spermatids are subjected to 16 steps of morphological changes to become mature spermatozoa [7–9].

There are two other models to describe SSCs self-renewal: A0/A1 model and A-dark and A-pale model. In A0/A1 model normal spermatogenesis is maintained by an “active” pool of SSCs (A1) and other quiescent “reserve” pool of SSCs (A0) is mobilized only following an insult to spermatogenesis [10]. In higher primates and humans two types of morphologically distinct SSCs are described,  $A_{dark}$  and  $A_{pale}$ . Observing biological functions of two cell types,  $A_{pale}$  is considered as progenitor cells and  $A_{dark}$  as true stem cells.  $A_{dark}$  represents only 1% of spermatogonia population, and stay dormant or divide very rarely if only progenitor cells have been destroyed.  $A_{pale}$  proliferate at defined periods during each cycle of the seminiferous epithelium and differentiate into B spermatogonia while leaving sufficient amount  $A_{pale}$  as functional reserve. In primates, single  $A_{pale}$  involve 5 mitotic divisions producing 32 spermatocytes and finally 128 spermatids. Amount of clonal expansion is very low in humans, and only 16 haploid cells are produced through 2 mitotic and meiotic divisions as depicted in **Figure 1** [11]. Due to low number of haploid cells produced by a single cell, both humans and primates maintain a population of progenitor cells ( $A_{pale}$ ) as a replenishment reserve. This is to minimize mitotic activity of true stem cells and preserve their genetic stability. Thus, the role of SSCs is to regenerate and sustain a cycling cell lineage, while progenitor population which is lacking regenerative capacity contributes to steady-state conditions [12]. There is no consensus on SSCs self-renewal in aforementioned models; whether it is through symmetrical (produce two stem cells or two interconnected cells destined to differentiate) or asymmetrical (produce one stem cell and other cell committed to differentiate). Using a mouse model, Wu

Mouse	A <sub>singl</sub>	A <sub>pair</sub>	A <sub>align</sub>	A1	A2	A3	A4	Int	B	Spc
No. of cells	1	2	4/8/16	16	32	64	128	256	512	1024
Rhesus monkey	A <sub>dark</sub>	A <sub>pale</sub>	B1	B2	B3	B4	Spc			
No. of cells	1	1	2	4	8	16	32			
Human	A <sub>dark</sub>	A <sub>pale</sub>	B	Spc						
No. of cells	1	1	2	4						

**Figure 1.** Pre-meiotic steps of spermatogenesis (SSCs to pre-leptotene spermatocytes) in different species of mammals. ■ Spermatogonial stem cells ■ Undifferentiated spermatogonia ■ Progenitor cells. Int – intermediate, Spc - spermatocytes.

et al. support the theory of asymmetrical division. Furthermore, they have proposed that fate decision of mammalian SSCs bifurcation is autonomous and stochastic [13].

Spermiogenesis is the process of transformation of spherical, haploid spermatids (n) to sperm-like mature spermatids. Human spermatid develops into a mature sperm through a series of 12 steps and it takes about 5 weeks. It is assumed that nuclear condensation during this process shuts RNA synthesis, and proteins required in the period (mainly protamine) are produced by stored mRNAs derived from the diploid phase of spermatogenesis [14]. Contrary to this suggestion, supportive evidences are emerging on the minor activity of transcription in haploid spermatids as well [15]. Spermiation is the last process involving breakage of the structures and bonds anchoring mature spermatids to Sertoli cells in order to release spermatozoa into the tubule lumen. Peristaltic waves created by peritubular smooth muscle cells help to move spermatozoa and testicular fluids through the seminiferous tubules to the epididymis [16]. This ~10–16 days migration through epididymis helps sperm to attain motility and natural fertilization capacity up to a certain extent [17, 18]. The total motility and fertilizability is gained only after mixing with accessory sex gland secretions [19].

**2.2. Regulatory mechanisms of natural spermatogenesis**

Number of sperm produced per day by testes (daily sperm production, DSP) is a tool for quantitative assessment of spermatogenesis. DSP can decrease with reduced amount of true stem cells present, failure to produce committed A<sub>pale</sub> cells, changes in niche environment due to multitude of causes, age (DSP is low in very young and older men), etc. [20]. However, even in the normal spermatogenic procedure germ cells may degenerate at various levels; pre-leptotene and leptotene spermatocytes in older men, and pachytene/diplotene spermatocytes across all ages. This would be a mechanism of eliminating cells with genetic abnormalities [21]. Other possible reason is to maintain the ratio of Sertoli cells to germ cells, as one Sertoli cell can assist only to a specific number of cells. Furthermore, there is no fine regulation of formation of spermatocytes in different areas of tubules, resulting unequal distribution of those cells. The apoptosis mainly involving the BCL-2 family of apoptosis regulating proteins helps to maintain an equal density of spermatocytes along the seminiferous tubule [22, 23].



In normal seminiferous epithelium, there is a well balance between SSCs self-renewal and differentiation. Loss of the equilibriums may cause either germ cell tumor or infertility subsequent to SSCs depletion. However, in specific situations, such as toxicity-induced spermatocytes destruction, this balance may be shifted toward differentiation over proliferation. One of the main regulators identified in the SSCs self-renewal is glial cell line-derived neurotrophic factor (GDNF) secreted by Sertoli cells [24]; whereas, well-known differentiation factors are stem cell factor (SCF) secreted by Sertoli cells and biologically active derivative of vitamin A; retinoic acid (RA).

### 2.3. Markers expression during spermatogenesis

Knowledge on cytological markers expressed or suppressed at different stages of spermatogenesis is a key factor on rapid development of *in vitro* spermatogenesis strategies. Several cytological markers such as genes, transcription factors, cytokines, growth factors, enzymes, other proteins and micro RNAs (miRNAs), etc., have been studied at distinct phases of spermatogenesis pathway from embryonic germ cells allocation to postnatal spermatogenesis process which is broadly divided into three phases; proliferation, differentiation, and spermiogenesis. Some of them are surface antigens found in different regions of the sperm and others are intracellular; in the nucleus or cytoplasm. Knowledge on the expression of stage-specific cytological markers is vital on studying germ cells biology, normal and abnormal pathways of spermatogenesis and deciding corrective measures. Proper process of spermatogenesis requires precise coordination of multitude of genes. Stage-specific surface markers may be involved in differentiation process at least part by interaction with Sertoli cells [25]. Surface antigens are extensively used for tracking subset of germ cells at specific differentiation stages, but the process is hampered as very few markers have been identified so far. Although most of these markers expressed on SSCs and early stages of differentiation, little or none of these antigens remaining on the head or tail of the sperm [26]. Majority of markers have characterized with animal germ cells, specifically using rat and mouse germ cells, and very few of them have tested in humans. We assume that the presence and behavior of majority of these genes are more or less similar between animals and humans.

#### 2.3.1. PGCs, SSCs, and spermatogonial progenitor cells (SPCs) markers

The chemokine receptor type 4 receptor (CXCR4) and DDX4 or mouse vasa homolog (MVH) genes are first expressed during migratory phase of PGCs, and MVH expression is continued until post-meiotic germ cells are formed. Decreased proliferative capacity of PGCs and defective spermatogenesis has been observed in MVH null mice. *PIWI*, *Fragilis*, *SSEA1*, and *STELLA* are other genes expressed in different levels in migratory PGSc. *Fragilis* is considered to be important for the migration of PGCs toward the genital ridges [27, 28]. B-lymphocyte-induced maturation protein-1 gene (*BLIMP1*) is involved in the initial specification of PGCs. Germ cells positive for *BLIMP1* proliferate continuously, and this process can be helpful to express other PGCs markers such as *Fragilis* and *STELLA*. A network of transcription factors are involved in maintaining embryonic properties of stem cells. *SALL4* is a member of Spalt-like transcription factor family, highly expressed in multiple embryonic tissues including PGCs and gonocytes. It is also involved in SPCs differentiation. Promyelocytic Leukemia Zinc Finger (*PLZF* or

*ZBTB16*) shows lower expression levels in embryonic germ cells and its peak in postnatal SPCs. It helps to maintain the properties of SPCs and also detected in early stages of SSCs differentiation. *SALL4* and *PLZF* physically interact and mutually oppose one another's localization to cognate chromatin domains depending on their relative expression levels at distinct stages of germ cells development. *c-KIT*, the transmembrane tyrosine kinase receptor for stem cell factor (SCF), also known as KIT ligand (KL) is essential to the proliferation and survival of differentiating spermatogonia, and its expression directly repressed by *PLZF*. At the time of differentiation, *SALL4* level increases with suppression of *PLZF* facilitating to expression of *c-KIT* [29]. *GPR125*, an orphan adhesion-type G-protein-coupled receptor, is another gene exclusively expressed in SPCs and SSCs, and not in differentiated spermatocytes. *GPR125* positive cells can be cultured in undifferentiated state with remarkable increase in their number [30]. A gene named transcriptional repressor inhibitor of differentiation 4 (*ID4*) was recently identified using a transgenic mouse model and it was highly expressed in most gonocytes, a subpopulation of SSCs, and a minor subset of pachytene spermatocytes [12]. But, they conclude the appearance of *ID4* in pachytene stage may be nonspecific, and proposed that *ID4* positive subpopulation may be a heterogeneous population of SPCs and SSCs in mouse. Another subset of rare and highly proliferative A<sub>single</sub> spermatogonia has been characterized in mouse by expression of the paired box transcription factor (*PAX7*). Using cell lineage tracing studies they have confirmed that *PAX7* cells function as bona fide stem cells [31]. The marker is co-expressed with well characterized other spermatogonial markers such as, *c-KIT*, *PLZF*, *FOXO1*, *RET*, and *GFRα1*. *PAX7* was reported perfectly conserved in 11 different species, and it is resistant to chemo and radiotherapy insults [32]. GDNF seems to stimulate SSCs self-renewal by signaling through *Ret* and *GFRα1* receptors system, and overexpression causes to an increase of undifferentiated spermatogonia in the testis [24]. POU family transcription factor 1 or octamer-binding transforming factor4 (*POU5F1/OCT4*) is expressed throughout the PGCs migration and later on in SSCs and differentiation male germ cells up to pachytene stage [5]. *Oct4* is rather considered as a pluripotency marker and expression is inhibited when RA binds to the responsive site, allowing the cells for differentiation. Many other markers expressed by SSCs and SPCs have described such as, *TERT*, *POU3F1*, *RBM*, *HSP90α*, *NGN3*, *NANOS2* & 3, *SOHLH1* & 2, integrin alpha chain 6 (*ITGA6/α6-Integrin/CD49f*), *LIN28*, *UTF1*, *CDH1*, *ITGB1* (*β1-Integrin/CD29*), *EPCAM* (*CD326*), *CD9*, *CD24* and *THY1* (*CD90*). *LIN28* and *EPCAM* are increasingly expressed in malignant germ cell tumors indicating their role as maintenance of cells in undifferentiated state. The *NANOS2* is also reported to block germ cells differentiation and lack of the gene induces progressive loss of germ cells in the postnatal testis [24, 28, 33–35]. New four marker genes specifically found in mouse PGCs and SSCs, but not in somatic cells were described as, *FKBP6*, *MOV10l1*, *4930432K21Rik*, and *TEX13* recently [36].

The above markers have been described using different techniques and most of them may have conserved among closely related animals during evolution. Results from RT-PCR analysis of freshly isolated human spermatogonia indicated that they are positive for *GPR125*, *GFRα1*, *PLZF*, ubiquitin carboxyl-terminal esterase L1 (*UCHL1*), and *RET* transcripts [37]. Using immunofluorescence and colorimetric staining it has been shown that human spermatogonia on the basement membrane express *UTF1*, *SALL4*, *ZBTB16*, *GFRα1*, *UCHL1*, *GPR125*, *LIN28*, *EXOSC10*, *FGFR3*, *DSG2*, *CBL*, *SSX2*, *OCT2*, *OCT4a/b*, *TERT*, *NANOG*, *ENO2*, and *PCNA* (a proliferation marker). Not like in rodents, *GPR125* is expressed only in subset

of SSCs in humans. SSCs expressing, epithelial cell adhesion molecule (*EPCAM*), *THY1* and *ITGA6* have been enriched using fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) techniques [38–41].

### 2.3.2. Differentiation markers

These markers can be categorized according to the timing of meiotic cycle; pre-meiotic, meiotic, and post-meiotic markers.

#### 2.3.2.1. Pre-meiotic

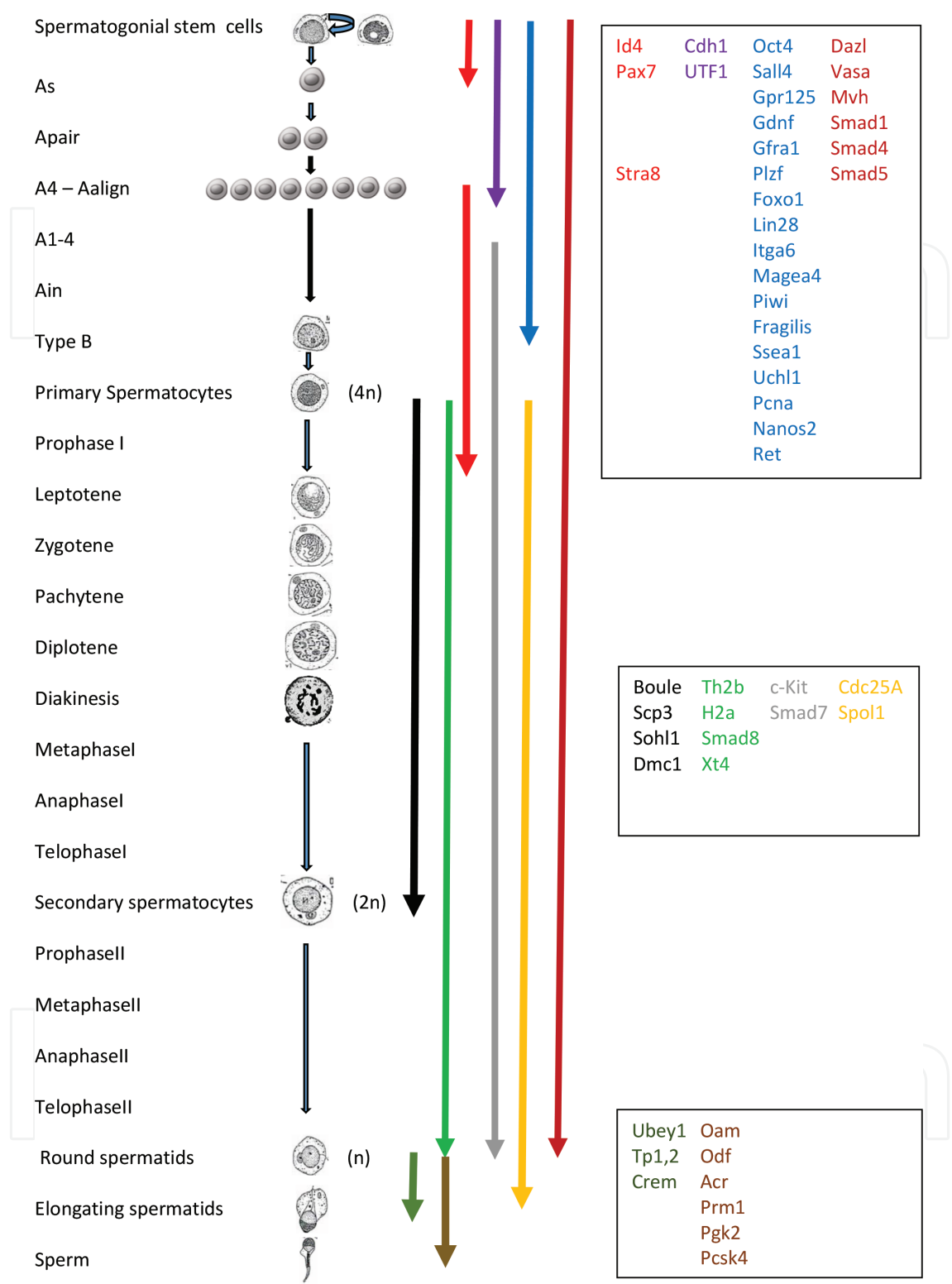
*c-KIT* is an early differentiation marker highly expressed in  $A_{al}$  spermatocytes onward. *c-KIT* and its ligand SCF or KL are involved in growth and survival of germ cells. Interaction between the SCF positive Sertoli cells and the *c-KIT* positive germ cells are helpful for the progression through meiosis. Cytochrome p450 family 26, sub family b, polypeptide1 (*CYP26b1*) gene is increasingly expressed in pre-meiotic germ cells. It may prevent spermatocytes enter into meiosis by blocking the action RA, which is one of the key signaling molecule that helps to induce meiosis by binding through nuclear RA receptors. Stimulated by retinoic acid 8 (*STRA8*) is the major responsive gene for RA induction and prominently expressed in pre-meiotic cells. At the same time RA receptors are expressed from type A spermatogonia to pre-leptotene spermatocytes in mice. Similarly, growth factor KL increases the percentage of meiotic entry in cultured spermatogonia concomitantly with an upregulation of *STRA8*. Activation of phosphatidylinositol 3 kinase (*PI3K*) signaling appears to be important in meiotic initiation of germ cells [33, 42, 43].

XT-1 is an adhesion related surface antigen found on differentiation spermatocytes in mouse testes. It is first detectable and uniformly distributed on leptotene spermatocytes, and later localized on the base of the head, tail and cytoplasmic lobe of the elongating spermatids [26]. Bone morphogenetic protein4 (*BMP4*) is an early differentiation marker, mainly activate through cell adhesion pathways and also upregulate *c-KIT* expression. It is prominently expressed in pachytene stage spermatocytes, and downstream proteins, SMAD1, 5, 8 are phosphorylated during *BMP4*-induced differentiation [17, 33]. SMAD1, 4, and 5 proteins are found in SSCs to round spermatids, while SMAD7 is found in differentiating spermatogonia up to round spermatids. SMAD8 is detected from spermatocytes to elongating spermatids [44]. Deleted in azoospermia-like gene (*DAZL*), *VASA*, *BLIMP1*, *STELLA* are also considered as pre-meiotic early germ cells differentiation markers [34]. Highest expression of *DAZL* is found in pachytene spermatocytes, and is assisted in the translation of MVH in the males. *Cyclin D2* is another gene expressed around epithelial stage VIII when the  $A_{al}$  spermatogonia differentiate into  $A_1$  stage [23].

#### 2.3.2.2. Meiotic

Specific markers well defined for meiotic germ cells are synaptonemal complex protein 3 (*SCP3*) gene and dosage suppressor of mck1 homolog (*DMC1*). Both *SCP2* and *SCP3* are components of the lateral element of the synaptonemal complexes and are associated with the centromeres in meiotic metaphase I cells. Another antigen named testicular differentiation antigen 95 (*TDA95*), residing on zygotene and early pachytene spermatocytes has been defined using two monoclonal antibodies, CA12 and BC7. *TDA95* may be one of the cell adhesion molecules between





**Figure 2.** Stage-specific germ cell markers compiled from different sources. Most of them are germ cell specific and some of the markers are common to both germ cell and few types of somatic lineage cells. These markers are significant in solid identification of germ cells at different phases of development.

spermatocytes and Sertoli cells, and plays an essential role during early meiotic prophase of spermatogenesis. *SCP1*, *CREST*, and *Tesmin* are other markers present in human pachytene spermatocytes. *Tesmin* expression coincides with meiotic entry of germ cells [25, 37]. *BOULE*

is a candidate meiotic regulator gene conserved among different species from drosophila to human, and deficient animals are infertile due to meiotic arrest in their male germ cells. The protein is detectable from pro-metaphase of first meiotic division to diplotene spermatocytes in humans and up to early spermatids in mice [45]. Similar expression pattern showed another meiotic promoter protein CDC25A, which was found in 1<sup>st</sup> and 11<sup>th</sup> spermatocytes and in some species up to elongating spermatids. Variations of three different isoforms of *BOULE* (*BOULE1*, 2, & 3) during spermatogenesis were described in humans [46]. *SPO11* (a type II topoisomerase), *H2A*, *TH2b* are other few genes, needed for meiotic recombination [43, 47].

#### 2.3.2.3. *Post-meiotic*

Expression of acrosin (*ACR*), transition protein 1 & 2 (*TNP1*, 2), ubiquitin-activating enzyme (*UBE1Y*), Kinesin light chain proteins (*KLC3*) and protamine1 & 2 (*PRM1*, 2) genes has been reported in post-meiotic cells [37]. *ACR* gene is transcribed in the diploid stage, and translationally expressed in the post-meiotic haploid cells. Hence, proacrosin is first localized in the cytoplasm of round spermatids. *PRM1* and *PRM2* are small and highly basic proteins that are reported to be transcribed in round and elongating spermatids. *KLC3* is a testis specific protein and acts as an anchor protein for binding mitochondria to outer dense fibers of sperm tail. During the spermiogenesis, somatic histones are replaced with testis specific nuclear proteins, termed as transition proteins (*TNP1* & *TNP2*), and subsequently *TNPs* are replaced with *PRMs*. Formation of extensive disulfide cross links between *PRMs* results in condensation of nuclear proteins and repression of transcriptional activity [48]. Alteration of *PRM* expression has been reported to affect human male fertility. For example, high susceptibility to DNA damage was reported with diminished levels [49]. Bone morphogenetic family proteins *BMP8a* and *BMP8b* receptors are expressed in male germ cells in a bimodal manner. First, low levels of transcripts are found in spermatogonia and primary spermatocytes, and subsequently higher levels are expressed in round spermatids. *BMP8b* is most important in initiation and maintenance of spermatogenesis [50]. **Figure 2** depicts stage-specific expression of few selected markers commonly found in literature.

### 3. Attempts to differentiate spermatozoa outside the niche (*in vitro*, *in vivo*, and *ex vivo*)

Understanding the components of SSCs niche and their interactions with each other are vital aspects in regeneration of spermatogenesis *in vitro*. The *in vivo* niche of mammalian SSCs is comprised of Sertoli cells, peritubular cells, and a complex array of matrix proteins. The normal SSC pool is maintained throughout adulthood, through signals provided by adhesion molecules and other cell surface receptors. SSCs are exposed to signals from both tubular lumen and the interstitial space sides of the basement membrane. Fate of SSCs is regulated mainly by Sertoli cells, inter-tubular blood vessels, and surrounding Leydig cells also have a role [51]. Steady state of germ cell niche can be disturbed by physiological changes of individual components by intrinsic or external factors. Regeneration of sperm has been difficult due to incomplete understanding of complex interactions within the niche environment, and germ cell-specific events such as, meiosis, chromatin re-modeling/repackaging,

flagellum development and transcriptional reprogramming, etc. [52]. However, some recent advancements in sciences, such as construction of “omics” databases involving genomics, proteomics, and metabolomics have immensely contributed to rapid development of germ cell biology. Studies have shown that thousands of genes are successively expressed or suppressed leading to changes in biochemical composition in germ cells along the spermatogenesis pathway. Epigenetic reprogramming of genes and post transcriptional modifications of proteins are further favored this process. In addition, miRNAs play a significant role in regulating germ cells differentiation [37]. Based on the available data scientists have developed *in vitro* culture systems to induce male germ cells development from different types of stem cells. The improved culture systems facilitate to study the distinct pattern of gene expression in germ cells at various developmental stages.

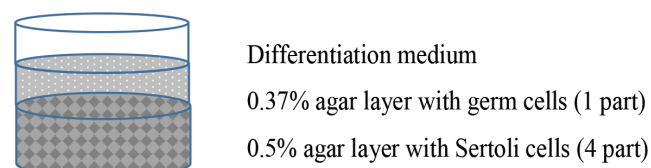
Recapitulation of spermatogenesis completely or as in part, outside its niche is essential to understand the series of biological events associated with this complex process. The techniques can be utilized to study the germ cell biology (mitosis, meiosis, morphogenesis, initiation of motility, etc.) toxicological studies, fertility preservation, production of transgenic sperm, and have the potential for new therapeutic approaches in male infertility [53]. Continuous attempts have been made using pre-existing immature germ cells or various sources of stem or somatic cells as the starting source for *in vitro* derived gametes with satisfactory results [54]. However, same weight can be given for the doubts still have to be clarified. The strategies are broadly categorized into three aspects; development of different culture systems, haploidization, and differentiation of germ or somatic cells, and autologous or xenologous transplantation of germ or putative germ cells.

Methods to isolate SSCs from testicular tissue and differentiate into haploid cells or further to sperm, with feeder or feeder free conditions have been explored in different studies. Enrichment of SSCs *in vitro* facilitates dissection of germ cells biology, because SSCs represent a very rare population of germ cells consisting about 0.03% (20,000–35,000) in adult mouse testes [5] or even lesser 2000–3000 [6]. Early attempt to germ cell culture (in which whole segment of seminiferous tubules were maintained in culture) goes back to 1964 [55]. The first human SSC culture was reported in 1998 using a crude extract of testicular tissue, and the efficacy has now been improved with varying techniques [56]. Digested testicular tissues from obstructive azoospermic men cultured with high concentrations of follicle-stimulating hormone (FSH) and testosterone continued the *in vitro* reduction of germ cell ploidy with rapid morphological changes toward spermiogenesis. Two different mechanisms are possibly involved in endocrine regulation of the above process, and they would be, prevention of Sertoli cells apoptosis by testosterone and stimulation of the spermiogenesis by FSH [57]. Following a simple two enzymatic digestion protocol *OCT4*<sup>+</sup> SSCs were isolated from human testis with 87% purity. SSCs colonies were able to culture for around 1 month on a Sertoli cells feeder layer [40]. In another study, *THY1*<sup>+</sup> mouse SSCs have been cultured in the presence of Sertoli cells, hormones (FSH/testosterone) and vitamins (RA/vit.E/C), either with a mix of three components or with individual components. After 7 days of culture spermatid-like cells expressing post-meiotic markers were prominent in mixed supplement group compared to individual supplements [47]. Addition of FSH to bovine SSCs culture has proven the increased colonization capacity of spermatogonia [58].

Differential plating is the simplest technique isolating germ cells from digested testicular tissues. However, a highly purified SSCs population is expected only from sorting of cells using combination of surface markers. The array of potential markers reported for isolating SSCs are *GPR125*, *ITGA6*, *CD9*, or *GFR $\alpha$ 1* [51]. Culturing of SSCs isolated using differential plating technique from testicular tissue of cancer patients, in laminin or gelatin coated wells (feeder free conditions) and serum free culture media supplemented with human GDNF, bFGF, EGF and LIF resulted in the increase of GPR125<sup>+</sup> cells from 2 to 70% [59]. In another study, human adult SSCs were able to culture in laminin coated plates, up to 28 weeks with 18,000-fold increased in number in the presence of LIF, bFGF, GDNF, and EGF [60]. In contrast, SSCs underwent massive apoptosis in feeder free conditions, but testicular somatic cells together with GDNF supported the propagation of SSCs more than 1 year [51].

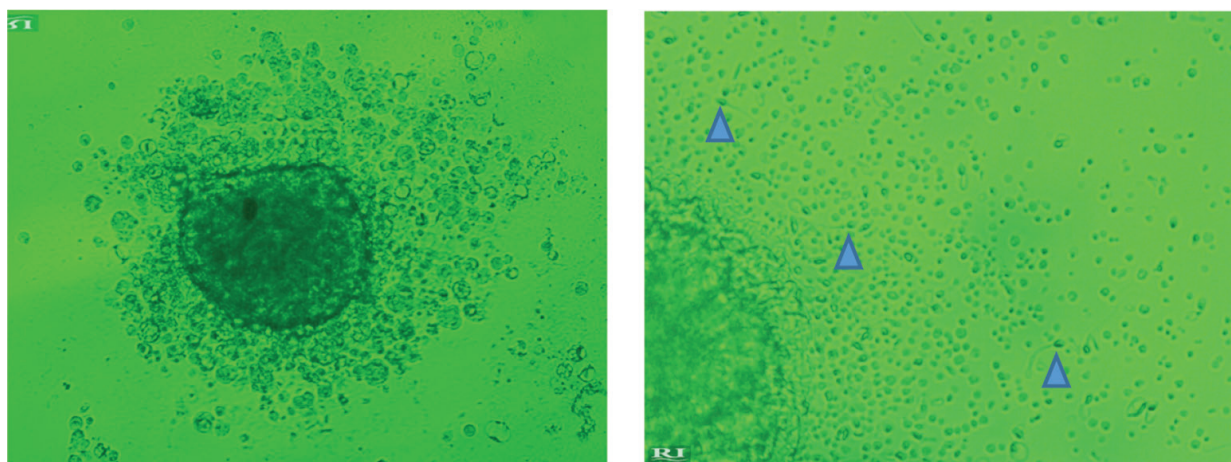
Maintaining the spatial arrangement of testicular cells seems to be important in the process of regulation and completion of spermatogenesis. The goal may be achieved by arranging germ and somatic cells in three-dimensional (3D) culture systems by formation of embryoid bodies (EBs) or culturing the cells in soft agar or methyl cellulose [61]. Two culture systems are depicted in **Figures 3** and **4**. The studies have been highlighted that, low temperature (equal to testicular temperature), endocrine factors, and supporting somatic cells are prerequisites to be considered in *in vitro* spermatogenesis. Supportive mechanisms provided by somatic cells to develop germ cells are controversial. Presence of somatic cells, but not necessarily the direct contact is suggested for *in vitro* proliferation of male germ cells in one study [61]. In contrast, significance of direct cell to cell contact between Sertoli cells and stem cells has been emphasized for successful germ cells formation from Warton's jelly-derived mesenchymal stem cells [62]. Reasons of such kind of variations, whether due to the source of cells used for differentiation of germ cells or specific stage of supporting along the differentiation process, should further be investigated.

Transmeiotic differentiation is one of the critical step in the spermatogenesis pathway, and it is inducible employing bio-mechanical or chemical methods such as, simulated gravity, KL, or RA. *c-KIT*<sup>+</sup> spermatogonia cultured under simulated microgravity for 48 h entered into meiosis even in the absence of exogenous supplements or Sertoli cells. Microgravity may act as an inducer or accelerator in the progression of meiosis [43]. Co-culture with testicular somatic cells, induction with RA and BMP4 are other well documented methods for meiotic initiation of SSCs. Mouse embryonic stem cells (ESCs) are reported to enter early meiosis when co-cultured with Sertoli cells compared to culture provided with RA. Sertoli cells provide RA for germ cells in two ways; by direct delivery of RA and delivery of retinol via membrane receptor *STRA6* [63]. Rate of germ cells formation and meiotic entry may also vary in



**Figure 3.** Schematic of soft agar 3D culture system.





**Figure 4.** SSCs co-cultured with Sertoli cells in embryoid bodies culture system. A—initial culture (day 5) showing spermatocytes and spermatids like stages. B—late phase of culture (day 14), few sperms with normal morphology are observed (arrow heads).

different culture systems, and with the source of cells used. For example, 3% of bone marrow mesenchymal stem cells (MSCs) were differentiated into germ cells when treated with RA [64]. Another study achieved around 20% of germ cells after co-culturing human ESCs with fetal gonadal stromal cells [65]. Mouse-induced pluripotent stem cells (iPSCs) treated with BMP4 led to formation of 41% primordial germ cells like cells [66]. However, very limited or pseudoentry of meiosis was noted by many studies. Around 3–5% of haploid cells were present in FACS sorted cells after induction of mouse SSCs in differentiation medium [67]. Similarly, 1–5% of post-meiotic cells were emerged when hESCs derived embryoid bodies (EBs) were treated with mouse testes conditioned medium supplemented with RA and BMP4 [68]. In contrast, 20% of haploid cells were observed in BMP4-induced human IPCs culture, and around 70% was positive for acrosin after sorting for 1 N cells [69]. Cell organization in EBs may reflect more of the arrangement of embryonic gonadal ridge [64], and correct erasure of imprinting genes was observed with EBs culture system [70]. It has been reported that, testosterone causes to increase in *STRA8* mRNA levels (pre-meiotic marker) when cells were treated with both RA and testosterone [71]. The spontaneous differentiation of stem cells into haploid state may also be possible in appropriate culture conditions with a very low efficiency, amounting around 2% in human iPSCs culture [69]. Spontaneous differentiation may increase with prolongation of culture, and due to inducing factors contained in culture medium. For example, media supplemented with 10% fetal bovine serum contain approximately  $3.6 \times 10^{-8}$  M of RA [72]. However, spontaneous differentiation of ESCs into germ line is generally low and inefficient with majority of germ cells undergoing degeneration [71].

Most of data have produced from very short period of cultures (2–30 days), indicating germ cell differentiation proceed an unusual speed *in vitro*. It is not clear how the timing went shorten, and it has been suggested that in the absence of environmental cues, germ cells may develop according to an intrinsic clock [73]. However, establishing a standard culture system of SSCs is difficult due to many reasons. Inherent variability between cells of different species leads to inconclusive results. For example, feeder cells and serum in culture may positively be



affected on some cell lines, but not on others. Batch to batch variations of serum and positive or negative impact of unknown factors added by both feeder cells and serum are also concern [56]. Many authors emphasize the requirement of refined culture system eliminating serum and feeder cells to better understanding of individual cellular and molecular interactions.

The *in vitro* maturation of available germ cells has a little value considering the therapeutic aspects of infertile men especially for men with non-obstructive azoospermia. To overcome this situation, ESCs derived gametes were tested as a first line remedial step by scientific community. Initial attempts were least successful due to unresponsiveness of cells for specific media or culture conditions [74]. However, encouraging models have been described later on by different authors, not only for ESCs but also for other multipotent cell types including iPSCs and somatic stem cells. Two main approaches for this are (a) direct differentiation of stem cells into germ cell lineage using exogenous factors; (b) transfection of stem cells with marked or fluorescent proteins linked to specific gene promoters, such as *STRA8* and *PRM1* [75]. These methods can be employed as monolayer adherent cell culture or three-dimensional embryoid bodies, with or without feeder cells. Optimum time point for obtaining germ-like cells from human pluripotent stem cells (PSCs) was recorded day 10, while day 7 cultures yielded lower numbers and day 15 not indicated a significant increase [67]. It is reported that germ cell formation in EB culture system seems to faster than in monolayer culture system [76]. Given the priority for direct differentiation method is more acceptable as gene transfection method disqualifies in clinical applications. However, the gene transfection and iPSCs systems may provide the necessary information on the behavior of related genes in germ cell development. Whatever the method employed, the imprinting regulation of gametes obtained from concerned methods has to be further validated, if ever they are to be used for clinical applications [73].

The possibility of using PGCs and germ line stem cells (GSCs) in transplantation studies to restore fertility has been studied with varying degrees of success [65]. Grafting or transplantation of gonadal fragments, germ cells or genetically modified germ cells and transmeiotic pluripotent stem cells onto immune compromised animals is an alternative strategy to investigate germ cell development. Successful autologous-transplantation of spermatogonial stem cells has achieved in a wide range of species so far [77]. Autologous cryopreserved testicular tissue grafting is an option for preserving genetic materials in endangered species and immature cancer patients. Success of homing ability of grafts may depend on various factors such as, age of collecting graft (immature is the better), low GnRH level (suppressed spermatogenesis with more primitive cells), method of cryopreservation, etc. [78]. Homing ability of SSCs from different species including human, to basement membrane of seminiferous tubules of nude mice has been proven by many authors [60]. The niche for spermatogonial proliferation appears to be generally similar among different species, because proliferation is undisturbed between cross-species after xenotransplantation of spermatogonia. However, the niche for spermatogonial differentiation is thought to work through a species-specific mechanism [78].

The most advanced progress in meiosis and qualified male gametes may be obtained following transplantation of *in vitro* derived PGCs or GSCs into the testis. The ability to develop more mature germ cells from PGCs like cells derived from mouse iPSCs has shown after

Source of cells	Method used	Observations	References
BM stem cells from <i>STRA8-EGFP</i> transgenic mouse line	Induced with RA (10 $\mu$ m) for 10 days. and EGFP positive cells were sorted using FACS	3% cells differentiated into male germ cells assessed by <i>OCT4</i> , <i>Fragilis</i> , <i>STELLA</i> , <i>MVH</i> , <i>RNF17</i> , <i>DAZL</i> , <i>c-KIT</i> , <i>PIWIL2</i> , <i>RBM</i> , <i>STRA8</i> , <i>TEX 18</i> . But arrested at pre-meiotic stage	Nyernia et al. [64]
hESCs (HSF1, HSF6 and H9) and hIPS	Co-cultured with human fetal gonadal stromal cells for 14 days	20% germ cells with triple positive markers ( <i>cKIT</i> , <i>SSEA1</i> & <i>PLAP</i> or <i>VASA</i> ). Repression of <i>HOX</i> genes and imprint erasure by day 7. No report of meiosis	Park et al. [65]
hESCs (H1) and iPSCs (HFF1)	Cultured in mouse SSCs differentiation medium for 10 days and haploid cells were confirmed after sorting by FACS	<i>UTF1</i> , <i>PLZF</i> and <i>CDH</i> positive spermatogonia, <i>HIWI</i> and <i>HILL</i> -positive spermatocytes, and <i>ACR</i> , <i>TP1</i> and <i>PRM1</i> expressing haploid cells (3.9–4.5%) were observed.  Unimpaired uniparental genomic imprints on two loci: <i>H19</i> and <i>IGF2</i>	Easley et al. [67]
iPSCs (iHUF4/IMR90) hESCs (H9/HSF1)	Induced with BMP-4, 7, and 8b for 14 days in feeder free conditions  Transduced with <i>VASA-GFP</i> reporter	Increased <i>Vasa</i> and <i>DAZL</i> expression. 4–6% GFP positive cells within 14 days.  Increased number of meiotic cells in <i>DAZL</i> overexpressed cells	Panula et al. [69]
<i>STRA8-EGFP</i> transfected Mouse ESC line (C57BL6)	Co-cultured with Sertoli cells or RA (on gelatin coated plates)	<i>DAZL</i> , <i>SYCP3</i> , <i>PRM</i> expression in both groups after 12 days of induction. Number of colonies and positive cells were high in Sertoli cell group	Miryounesi et al. [63]
hESC lines (Shef1–6 & H7)	EBs were induced with RA, <i>Bmp4</i> and neonatal mouse testis conditioned medium for 14 days	Progressive elevation of both spermatogenesis and oogenesis markers. Effect was prominent with RA treatment. 1–5% post-meiotic cells & few with the beginning of flagellum formation	Aflatoonian et al. [68]
Mouse C2C12 myoblast cells	<i>STRA8</i> -positive C2C12 myoblasts were treated with 10 $\mu$ M all-trans-RA for 8 days	Pre-meiosis, meiosis and post-meiosis gene markers were expressed. Few cells exhibited spherical morphology with tail like structure. But the cells were diploid indicating arrest at pre-meiotic stage	Jia et al. [35]
Pre-meiotic male germ cells from immature mouse	Cells were cultured in gel matrices (soft agar or methyl cellulose) with the support of somatic cells and gonadotropins for 40 days	Morphologically normal but immotile spermatozoa	Stukenborg et al. [61]

Source of cells	Method used	Observations	References
Stella-GFP <sup>+</sup> ES cells	Cells were cultured in Ham's F12/IMDM medium with BMP4 as adherent or EBs culture systems	Cells in both cultures, predominantly in EBs were differentiated, into primordial germ cells with correct gene expression patterns. Correct pattern of parental imprint erasure was confirmed ( <i>PEG3</i> and <i>IGF2R</i> )	Wei et al. [70]
Porcine skin-derived somatic stem cells	Induced with porcine follicular fluid for 50 days	Large, round PGCs like cells with 0.25% positive for alkaline phosphatase (AP). Cells were positive for <i>c-KIT</i> , <i>OCT4</i> , <i>VASA</i> , <i>STELLA</i> , <i>DAZL</i> . 99% of CpG sites were unmethylated in <i>DMR1</i> of PGCs	Linher et al. [86]
Human ESCs (HSF-6 & H-9)	50–250 cell colonies were cultured on mouse primary embryonic fibroblast in ESCs growth medium for 7d. The cells were co-cultured with putative Sertoli cells	AP & <i>CXCR4</i> , <i>c-KIT</i> positive primordial germ cells arose. Electron microscopic pictures showed large round nucleus with numerous mitochondria	Bucay et al. [87]
Mouse SSCs from 7d old male	3D agar culture system consisting 0.5 agar and 25% FCS in lower layer and 0.37 agar and 20% FCS in upper layer. Culture continued for 30d with PRMI media	Different stages of spermatogenesis were observed with increasing meiotic and post-meiotic markers with time. Average 15 spermatozoa per well of 24 well plate were present	Elhija et al. [88]
Laminin binding spermatogonia from Sprague-Dawley rats	Cells were cultured in gelatin coated wells with a serum free formulated medium for 120 days. Other supplements were GDNF and FGF	Cultured SSCs effectively regenerated spermatogenesis in testes of busulfan-treated recipient rats	Wu et al. [89]
Immature spermatogenic cells isolated from non-obstructive azoospermic men	3D culture in a collagen gel matrix with somatic cells. The media supplemented with RA and rFSH	More round and elongating spermatids emerged at day 12 of culture. <i>PRM2</i> + ve cells and haploid cells were increased with time	Lee et al. [90]
<i>in vitro</i> generated germ cell line from teratocarcinoma cells (F9)	TC cells were transfected with <i>Stra8-EGFP</i> fusion construct and positive cells were induced with RA. FACS sorted germ-like cells were transplanted.	Mature sperm after 7 months of transplantation in recipient mice testes. Performance of ICSI confirmed the oocytes activating capacity of these sperm	Nayernia et al. [91]
TESE dissociated <i>CD49f</i> positive cells from azoospermic men	Cells were cultured with Sertoli cells and culture media containing RA, GDNF, FSH & testosterone for 15 days	Progression of meiosis up to day 5. Correct gene expression pattern ( <i>SCP3</i> and <i>CREST</i> and haploid cells were obtained	Riboldi et al. [92]

Source of cells	Method used	Observations	References
Human Wharton's jelly-derived MSCs	MSCs were co-cultured with mitotic inactivated newborn mouse Sertoli cells for 3 weeks	Typically round germ-like cells were appeared with time. The cells were positive for early germ cell specific <i>STELLA</i> & <i>VASA</i> and male germ cells specific <i>DAZL</i> markers	Xie et al. [62]
Human testicular tissues from first and second trimester fetuses	Testis tissues were inserted subcutaneously into mice. Mice with second-trimester xenografts were randomly given hCG injection	Completed the normal seminiferous cord formation. Induction of steroidogenesis was observed with hCG treatment. Germ cells differentiation was confirmed by decreasing <i>OCT4</i> and increasing <i>Vasa</i> expressing cells	Mitchell et al. [93]
MSCs from human umbilical cord Wharton's jelly	Cells were induced with $2 \times 10^{-6}$ M RA and 10 ng/ml BMP4 in DMEM for 7–14 days	Small number of germ cells expressing <i>SCP3</i> and <i>VASA</i> were formed	Hua et al. [94]
Mouse ESCs	ESCs derived PGCs like cells were cultured with testicular somatic cells in the presence of Knockout serum, RA, BMP 2, 4, 7, Activin A, FSH, Testosterone and bovine pituitary extract for 14 days. Resultant spermatids like cells were used for ICSI	Confirmed meiosis in 84% of colonies and erasure of genetic imprinting. ICSI procedure using spermatids like cells produced viable and fertile offspring	Zhou et al. [95]

**Table 1.** A summary of selected studies designed to differentiate male germ cells from different sources of stem cells.

transplantation into testis of infertile mice [66]. Nayernia et al. reported 300–500-fold increase in spermatogonial population after transplantation of mouse bone marrow (BM) derived SSCs into germ cells depleted recipient mice, but the cells were arrested at pre-meiotic stage [64]. A similar study has shown that transplanted green fluorescence protein positive (GFP<sup>+</sup>) mouse BM cells can differentiate into both somatic and germ cell lineages in a favorable testicular niche [79]. Transplanted adipose tissue-derived MSCs could induce spermatogenesis in busulfan-treated recipient rats in another study [80]. Toyooka et al. cultured *Mvh* knock-in GFP or *Lac-Z* mouse embryoid bodies with *BMP 4,8* expressing embryonic trophoblast cells, and they could achieve morphologically normal sperm after transplantation of *MVH* overexpressing cells under the testis capsules of nude mice [81]. Use of combination of gene transfection and subsequent germ cells transplantation techniques could results live sperm (with reduced motility) and offspring after intracytoplasmic sperm injection (ICSI) in mice. Though the newborns died within few months due to imprinting defects, this finding paves the way to promising *in vitro*- and/or *ex vivo*-derived functional gametes in the future [82]. Additional treatment with hormones (hCG) after stem cell transplantation may be a powerful tool for increasing the efficiency of transplantation [83]. It is suggested that FSH and testosterone favor the survival of germ cells by regulating both intrinsic and the extrinsic apoptotic pathways. Furthermore, FSH is needed to initiation of meiosis and androgen is necessary for the completion of meiosis and spermiogenesis [84].

Adult somatic cells induction (SCI) and sperm cloning (male genome cloning) are recent advancements and future directions for generating clinically applicable germ cells from stem cells. In SCI technique, somatic cell nucleus is injected into enucleated oocyte and cells are cultured further to produce two separated chromosome sets. Thus, the immature oocyte helps somatic cell to become haploid. The resultant cells are genetically identical and immune-compatible with the donor of the somatic cells. In case of severe oligozoospermia, a single viable sperm from testicular biopsy sample can be used for replicating its genome. Here, a single sperm is injected into enucleated oocyte and allows it to become a haploid embryo dividing through parthenogenesis process. Resulting blastomeric cells may be used for further *in vitro* maturation or direct injection into oocyte. These new techniques still remain in experimented animal models with low efficiency [85]. The contribution by various authors for development of this field is summarized in **Table 1**.

#### 4. Conclusion

Among the different models used in *in vitro* spermatogenesis better results have been achieved through 3D culture systems. Formation of EBs using germ and Sertoli cells seems to be more efficient and resemble the testicular niche environment compared to soft agar culture system. Although this system is well supported for the proliferation and differentiation of putative germ cells obtained from somatic stem cells up to the spermatids state there is no firm evidence to conclude the support for an efficient spermiogenesis process. Complete spermatogenesis has been achieved by transplanting stem cells or putative germ cells into testes in few studies, but with lower efficiency. Clinical applications of xeno or autologous transplantation studies among humans, and between humans and animals are still far away due to ethical and safety related issues. Similarly, use of autologous stem cells for differentiation of germ cells and infertility treatment may have little value for the patients with cancer or genetic diseases, as there is a possibility to re-infuse cancer cells or passing genetic abnormalities to offspring. However, *in vitro* maturation of germ cells may be immensely helpful for men with maturation arrest or azoospermia due to non-genetic causes.

#### Abbreviations

AP	alkaline phosphatase
ART	assisted reproductive technologies
bFGF	basic fibroblast growth factor
BM	bone marrow
DMEM	Dulbecco's modified eagle medium
DSP	daily sperm production
EBs	embryoid bodies
EGF	epidermal growth factor



EGFP	enhanced green fluorescence protein
ESCs	embryonic stem cells
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
GDNF	glial cell line-derived neurotrophic factor
GSCs	germline stem cells
ICSI	intra cytoplasmic sperm injection
iPSCs	induced pluripotent stem cells
LIF	leukemia inhibitory factor
MACS	magnetic-activated cell sorting
miRNA	microRNA
MSCs	mesenchymal stem cells
PGCs	primordial germ cells
RA	retinoic acid
SPCs	spermatogonial progenitor cells
SSCs	spermatogonial stem cells
TESE	testicular sperm extraction
3D culture	three-dimensional culture

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