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Review Article

Regulation of Spermatogonial Stem Cell Compartment in the Mouse Testis

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Abstract

Spermatogenesis occurs throughout the adult lifetime of males and is supported by a robust stem cell system. Spermatogonial stem cells (SSCs) are the stem cells of postnatal male germ cells, and not only self-renew but also produce differentiated progeny continuously. Recent report revealed that differentiating spermatogonia could revert into an undifferentiated state, although it was believed that SSCs were homogeneous and that differentiating spermatogonia was not reversible. Although several molecules, which regulate SSC, have been identified so far, molecular mechanisms underlying the maintenance of SSCs as well as the reversible developmental lineage of SSCs remain to be elucidated. In this review, we describe a brief overview of spermatogenesis and summarize the molecular regulation of SSC compartment.

1. Spermatogenesis and spermatogonial cyst formation

Stem cells are defined by their function to generate multiple differentiated cell types and to retain the ability to maintain them in an undifferentiated state. This ability to self-renew supports the maintenance and the regeneration of the anatomy and function of an adult tissue. Spermatogonial stem cells (SSCs) are stem cells of male germ cells and support spermatogenesis throughout the adult lifetime of a male, because SSCs can maintain themselves and also produce differentiated progeny continuously[1)2)].

Spermatogenesis occurs in seminiferous tubule of the testis and is consisted of three distinct phases of differentiation stages. First phase is the proliferative phase, in which spermatogonia undergo mitotic divisions and germ cells expand their numbers. After some mitotic divisions, spermatogonia differentiate into spermatocytes, that germ cells enter the second phase, the meiotic division. Spermatocytes produce four haploid germ cells, spermatids, by meiosis, that germ cells enter the third phase of spermatogenesis, termed spermiogenesis. In the third phase, germ cells change their morphology from round spermatid to spermatozoa via elongating spermatids by rearrangement of cytoskeletal structure and condensation of chromatin followed by replacement of nuclear protein from histones to protamins. SSCs are the small subset of spermatogonia, which are located on the basement membrane of the seminiferous tubules.

During spermatogenesis, all germ cells are connected with neighboring sister germ cells by a unique structure called the intercellular bridge. Most primitive spermatogonia exist as A_{single} (A_s, isolated and single spermatogonia). As germ cells begin to differentiate, they form A_{paired} (A_{pr}, two spermatogonia connected by an intercellular bridge) and $A_{aligned}$ (A_{al}, 4, 8, or 16 spermatogonia connected by intercellular bridges). Spermatogonia connected in chains longer than 16 are believed to be committed to differentiation[3)4)]. The intercellular bridges are essential structure for spermatogenesis. When TEX14, which is responsible for the formation and the maintenance of intercellular bridges, is disrupted, spermatogenesis is impaired due to the defect of the differentiation of spermatogonial stem cells[5)]. Since the

chain formation through intercellular bridges is maintained throughout spermatogenesis, visualization of intercellular bridges allows one to distinguish the early stages of postnatal spermatogenesis as spermatogonia initiate the process of differentiation[6]. In the primate, there are two types of spermatogonia, A_{dark} and A_{pale} spermatogonia. A_{dark} spermatogonia function as reserve stem cells that rarely divide and replenish progenitor cell compartment in case of injury or disease, whereas A_{pale} spermatogonia are progenitors, in which germ cells expand their numbers by mitotic proliferation[7,8]. Relationship between the length of spermatogonial chains and differentiation status has not been clarified yet. In the classical "A_s model", A_s spermatognia is considered to be stem cells[9,10]. A_s spermatogonia undergo symmetrical division and generate either two A_s spermatogonia or one A_{pr} spermatogonia, which are committed to differentiation and loose stem cell function irreversibly. Although this concept is logically established on the basis of the morphological observation, it lacks actual behavior of stem cells. After establishment of spermatogenic cell transplantation technique, it has been demonstrated that the stem cell activity is enriched in the "undifferentiated spermatogonia", which include A_s, A_{pr}, and A_{al} spermatogonia[11]. "Differentiating spermatogonia", which include A_{1-4}, In, and B spermatogonia, are derived from "undifferentiated spermatogonia" and proceed to highly organized differentiation process.

2. Regulation of spermatogonial stem cells

In the last two decades, a number of genes have been shown to be involved in the regulation of SSCs. An extrinsic factor that regulates self-renewal of SSCs is glial cell line-derived neutrophic factor (GDNF), which is produced by Sertoli cells. GDNF stimulates SSCs to self-renew by signaling through a heterodimer of tyrosine kinase, RET and GDNF receptor family alpha 1 (GFRA1)[12]. In testes heterozygous for GDNF null mutation, spermatogenesis is impaired by defects in self-renewal of SSCs, whereas overexpression of GDNF induces an increase of undifferentiated spermatogonia in the testes[12]. When wild-type SSCs are transplanted into GDNF-null testes, SSCs cannot be maintained and disappear from the transplanted testes[12]. Expression of GFRA1 is restricted in most primitive stages of undifferentiated spermatogonia (A_s and A_{pr}) and used as a useful marker to distinguish undifferentiated spermatogonia[13,14]. Basic fibroblast growth factor (bFGF) is also an important extrinsic factor that can enhance self-renewal of SSCs in cooperation with GDNF, although bFGF alone is not enough for SSCs to self-renew[15]. Colony-stimulating factor 1 (CSF1) can also enhance self-renewal of SSCs in the presence of GDNF and bFGF[16]. On the other hand, Kit ligand (KITL) is an essential extrinsic factor for spermatogonia to differentiate[17]. Undifferentiated spermatogonia are accumulated in the testes of mice mutant for KITL. A tyrosine kinase receptor KIT, which is the receptor for KITL, is expressed in differentiating spermatogonia but not in undifferentiated spermatogonia.

The most important intrinsic factor is promyelocytic leukemia zinc finger (PLZF ; also known as ZBTB16 and ZFP145). PLZF, which is a transcriptional repressor, regulates transcription by negative cooperation with histone deacetylase (HDAC) and the co-repressors[18,19]. The expression of PLZF is restricted in undifferentiated spermatogonia. In PLZF mutant testes, spermatogonia exists at birth and spermatozoa are produced in the first round of spermatogenesis, but ultimately all germ cells gradually disappear from the testes. PLZF represses the expression of KIT in the undifferentiated spermatogonia and opposes activity of mammalian target of rapamycin complex 1 (mTORC1), which is a key mediator of cell growth, by induction of the mTORC1 inhibitor, Redd1[20,21]. Hyperactivation of mTORC1 leads to stem cell depletion and mTORC1 impairs SSC maintenance by a negative feedback to GDNF receptors. In differentiating spermatogonia, PLZF physically interacts with a pluripotency factor, Sall-like 4 (SALL4), and is displaced from noncognate chromatin to induce expression of KIT[22].

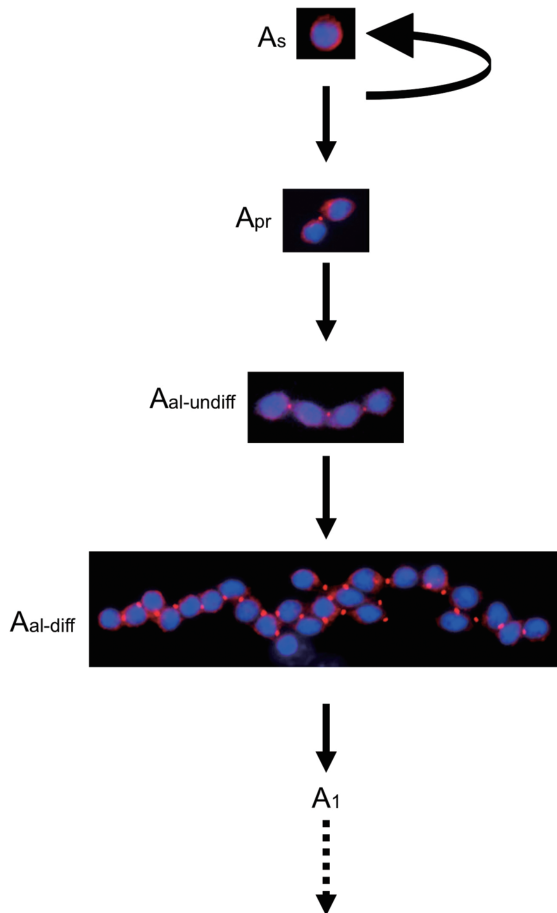

Fig. 1 Scheme of classical "As" model of spermatogonial differentiation and stem cell renewal as originally proposed by Hukins (1971) and Oakberg (1971). Immunostainings of TEX14 (red) and DAPI are shown. TEX14 positive intercellular bridges allow us to distinguish differentiation status of spermatogonial cysts.

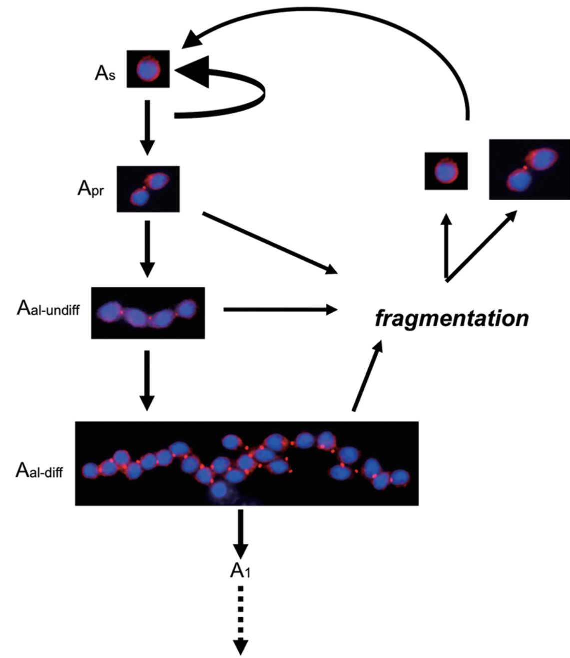

Fig. 2 Scheme of updated spermatogonial differentiation and stem cell renewal proposed by Nakagawa *et al*., (2010). Spermatogonial compartment is supplied by not only self-renewal of SSCs but also fragmentation of differentiating spermatogonia, which suggests dedifferentiation of differentiating spermatogonia.

Some genes related for pluripotency are also expressed in undifferentiated spermatogonia. Octomer-4 (OCT4 ; also known as POU domain class 5 transcription factor [POU5F1]) is expressed in undifferentiated spermatogonia and is used as a marker for SSCs[23]. When OCT4 is downregulated in SSCs, the SSCs cannot colonize in culture and transplanted testes without effect of expression of PLZF[24]. OCT4 may have different function from PLZF to maintain SSCs in an undifferentiated state. LIN28, which regulates miRNA maturation, is specifically expressed in undifferentiated spermatogonia. LIN28 is used as a marker of undifferentiated speramtogonia, although the role of LIN28 in SSC regulation still remains to be elucidated[25].

A zinc finger motif contained RNA-binding protein, NANOS2, is specifically expressed in early stages of undifferentiated spermatogonia (A_s and A_{pr}) in the testes and is also an essential intrinsic factor for the maintenance of SSCs[26]. The lack of NANOS2 in postnatal spermatogenesis induces progressive loss of germ cells within four weeks by the defect in the self-renewal of SSCs. When NANOS2 expression is forced in male germ cells, spermatogonia are prevented to enter into meiosis. There are few KIT positive differentiating spermatogonia and increased number of PLZF positive undifferentiated spermatogonia in the NANOS2 overexpressed testes, indicating that NANOS2 blocks differentiation of SSCs. Expression of NANOS2 is reduced by the lack of GFRA1 in the postnatal testes, suggesting that expression of NANOS2

could be regulated by GDNF signaling[27]. Overexpression of NANOS2 in the GFRA1 null testes prevents precocious differentiation of GFRA1 deficient SSCs and partially rescues loss of SSCs, although NANOS2 overexpression does not induce GFRA1 expression. The molecular function of NANOS2 has been shown to regulate target mRNAs posttranscriptionally. NANOS2 interacts with specific mRNAs as well as CCR4-NOT deadenylation complex and may promote the degradation of NANOS2-interacting mRNAs by recruitment of deadenylation complex[28]. Further analyses about the molecular mechanisms of NANOS2 as well as NANOS2 interacting mRNAs may provide clear insight into the regulation of SSCs.

A basic helix-loop-helix (bHLH) transcription factor, neurogenin 3 (NGN3), localizes in undifferentiated spermatogonia[29]. Since NGN3 starts to express in spermatogonia after birth and is not expressed in PGC, NGN3 is used as a marker to visualize spermatogonial chain formation[14,30]. Although molecular function of NGN3 in SSCs still remains unclear, NGN3 may be involved in differentiation of spermatogonia, because NGN3-negative undifferentiated spermatogonia shows high stem cell potential. Spermatogenesis and oogenesis specific bHLH 1 (SOHLH1) and SOHLH2 are co-expressed in the entire spermatogonia except for GFRA1 positive spermatogonia and are essential for differentiation of spermatogonia. Lack of SOHLH1 disrupts differentiation of spermatogonia, and undifferentiated spermatogonia do not differentiate into KIT-positive spermatogonia in the SOHLH2 null testes[31~33]. SOHLH1 and SOHLH2 are cooperatively suppress genes related for SSC maintenance, such as GFRA1, and induce genes important for differentiation of spermatogonia, such as KIT[34].

3. Reversibility of spermatogonial compartment.

It was believed that SSCs were homogeneous and that differentiating spermatogonia was not reversible, although it is well known in the *Drosophila* germline stem cell system that differentiating germ cells can revert into stem cells by abscission of intercellular bridges[35,36]. However, three research groups demonstrated that differentiating mouse spermatogonia could dedifferentiate into undifferentiated spermatogonia. Although KIT positive spermatogonia are differentiated and do not form colonies in the transplanted testes, they can revert into KIT negative undifferentiated spermatogonia by culture with GDNF and bFGF[37]. Another finding about reversion of differentiating spermatogonia into an undifferentiated state was provided by a live-imaging study. The combination of live-imaging and lineage-tracing experiments showed that spermatogonial cysts have the capacity to fragment, allowing A_{al} differentiating spermatogonia to repopulate the A_s and A_{pr} undifferentiated spermatogonia[14]. It has been also shown that the SSC compartment is rapidly and stochastically replaced by fragmentation of neighboring differentiating spermatogonial cysts along the seminiferous tubule[38]. However, molecular mechanisms underlying the fragmentation of spermatogonial cysts and dedifferentiation of spermatogonial compartment remain unclear.

Recently we found that loss of a histone demethylase, JMJD3, in male germ cells induces frequent fragmentation of spermatogonial cysts[39]. Among a number of epigenetic modifications, which include DNA methylation and histone modifications, methylation of lysine 27 of histone H3 (H3K27) is implicated in embryonic development as well as stem cell differentiation. H3K27 can be methylated by Enhancer of Zest Homologue 2 (EZH2, also called KMT6), a catalytic component of polycomb repressive complex 2 (PRC2), and is associated with transcriptional repression. Most targets of PRC2 are genes essential for development in embryonic stem cells, stem cell maintenance, and pluripotency[40,41]. Loss of any core components of PRC2 subunits (EZH2, EED, or SUZ12) results in a developmental block at the gastrula stage[42]. Although PRC2 components suppress differentiation of stem cells, they are not required for stem cell maintenance[43,44]. H3K27 methylation can be removed by UTX and JMJD3 (also called KDM6A and KDM6B, respectively).

UTX is ubiquitously expressed and escapes X-inactivation and regulates HOX gene activation and posterior development[45)46)]. Moreover, recent studies show that UTX is involved in myogenesis, cardiac development and epigenetic reprogramming[47)48)]. Alternatively, JMJD3 is predominantly expressed in stem cells and regulates differentiation and dedifferentiation in neural and epidermal differentiation, skin repair, inflammation, and epigenetic reprogramming[49)~52)].

We found that JMJD3 was specifically expressed in PLZF-positive undifferentiated spermatogonia in the testes[39)]. Down-regulation of JMJD3 in SSCs promotes an increase in undifferentiated spermatogonia but does not affect their differentiation. Germ cell-specific JMJD3 deficient mice have larger testes and sire offspring for a longer period than controls, likely secondary to increased and prolonged maintenance of the spermatogonial compartment. Moreover, the lack of JMJD3 induces frequent fragmentation of spermatogonial cysts by abscission of intercellular bridges, suggesting that JMJD3 controls spermatogonial compartment by the regulation of fragmentation of spermatogonial cysts. Because there has not been shown any molecules that regulate fragmentation and dedifferentiation of spermatogonial compartment, analyses of the molecular mechanisms underlying frequent fragmentation of spermatogonial cysts by the lack of JMJD3 could provide clear insights onto the regulation of SSCs.

Although expression of JMJD3 in human testes has not been identified, JMJD3 could be expressed in spermatogonia in the primate spermatogenesis. Although formation of intercellular bridges as well as chains of spermatogonia in the primate spermatogenesis has to be characterized well to apply rodent results, there are TEX14-positive intercellular bridges in primate spermatogenesis and primate spermatogonial cysts could be fragmented by abscission of intercellular bridges. Therefore, JMJD3 may regulate the fragmentation of spermatogonial cysts from A_{pale} spermatogonia to A_{dark} spermatogonia in the primate. Inhibition of JMJD3 to expand the number of spermatogonia *in vivo* and/or *in vitro* could improve infertile patient, because small molecule, which has been shown to inhibit JMJD3 activity, can be applied to the treatment[53)].

Somatic cells can be reprogrammed into a pluripotent state by induction of only four transcription factors[54)~58)]. Since various conflicts about moral issues and immunotolerance can be excluded by induced pluripotent stem (iPS) cells, research about iPS cells is a major topic of regenerative medicine[59)]. On the other hand, SSCs can obtain pluripotency in culture *in vitro*[60)~62)]. Intriguingly, SSCs express all of the genes that are required for induction of pluripotency (*i.e. Oct4, Sox2, Klf4, Myc,* and *Lin28, Nanog*). It is probably why SSCs can convert into pluripotent stem cells just in culture. However, the mechanisms underlying the reprogramming of SSCs into a pluripotent state still remain unclear. Although there are many similarities between SSCs and pluripotent stem cells, complete knowledge is still limited despite accumulating information about transcription network of SSCs after establishment of spermatogonial stem cell transplantation and generation of germ cell specific knockout mice. The understanding of the regulatory mechanisms of self-renewal, differentiation, dedifferentiation, and reprogramming of SSCs may provide unexemplified insights into not only self-renewal of other stem cells but also reprogramming of somatic cells.

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(References with numbers in bold are listed as important ones for readers.)

（和文抄録）

マウス精巣における精子幹細胞集団の制御

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精子形成は精細間の中で厳密に制御されており，体細胞分裂を行う精原細胞，減数分裂を行う精母細胞，そして半数体となり成熟精子へと形態形成を行う精細胞の主に三つの分化段階に分けられる．雄成体のほぼ一生にわたり続く精子形成は幹細胞システムにより支えられており，精原細胞のごく一部にある精子幹細胞が，このシステムの幹細胞である．精子幹細胞は自己複製と分化を繰り返すことにより，絶え間ない精子形成を保証している．

精子形成期にある生殖細胞は細胞分裂を経ても完全に分裂せずに細胞間架橋（Intercellular Bridge）により，つながれたまま，同調的に分裂増殖していく．細胞間架橋は精子形成において重要な構造体であり，欠損させると，精子幹細胞以降の分化が障害される．また，細胞間架橋を欠損した精子幹細胞は自己複製し，維持されるが，分裂に伴い細胞周期進行が阻害されるとともに，アポトーシスにより失われていく．精子幹細胞は均一な細胞集団であり，分化しつつある精原細胞は不可逆的に分化の運命を進行すると考えられていたが，最近の報告により，分化しつつある精原細胞が細胞間架橋を切り離すことにより，より未分化な状態に逆戻りできることが明らかとなった．すなわち，精子幹細胞集団は幹細胞自体の自己複製によってだけでなく，分化しつつある精原細胞からの補充によっても維持されていることが示唆され，古典的な考えが修正されつつある．

細胞間架橋の断片化による精子幹細胞の補充という新しい概念が登場した精子幹細胞システムについて，本稿では，これまでに同定された精子幹細胞の未分化性維持や分化に関わる分子群について，最新の知見も合わせて概説する．