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Review

Recent advances on identification of spermatogonial stem cells and their niche

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Abstract: Spermatogonial stem cells (SSCs) are the most primitive spermatogonia in the testis. A balance between the self-renewal and differentiation of SSCs, strictly controlled in a special 'niche' microenvironment in the seminiferous tubules (SSC niche), is essential to maintain normal spermatogenesis. Since the 1950s, many experimental methods, including histology, immunostaining, whole-mount analyses, and pulse-chase labeling, had been used in attempts to identify SSCs. Recent studies demonstrate that A_{undiff} seem to possess variable levels of stem cell potential to act as SSCs; $GFR\alpha1^+$ population in A_s has the greatest potential to act as SSCs (can consider as actual SSCs) whereas $NGN3^+$ population in A_{undiff} has comparatively much lower potential to act as SSCs (can consider as potential SSCs). The precise identity of SSCs is still being refined. Sertoli cells, directly interact with SSCs, and interstial cells including Leydig cells, testicular macrophages, peritubular myoid cells and vascular smooth muscle cells, control the proliferation and differentiation of SSCs through the secretion of extrinsic factors, constitute the cellular components of SSC niche which preferentially locates in the region of seminiferous tubules adjacent to the interstitium that always coincide blood vessels.

Keywords: SSC; niche; GFRα1; NGN3; vasculature

1. Introduction

Spermatogenesis is a complex biological process producing male haploid germ cells (sperm) from diploid SSCs, which maintain themselves (self-renewal) and continue to produce progeny that will differentiate into sperm over a long period (Cheng, 2008). Pioneering studies largely by extensive morphological analyses were conducted from the 1950's to the 1970's and have revealed the features of mammalian spermatogenesis and SSCs. The most widely accepted model is the 'A_{single} (A_s) model', which was originally established in 1971, is now prevailing: SSCs are regarded as a subset of undifferentiated type A spermatogonia (A_{undiff}) and isolated type A spermatogonia (A_s) are SSCs (Huckins, 1971; de Rooij, 1973, 2001; Meistrich and van Beek, 1993; Chan *et al*., 2014). In rodents, A_s undergo successive several mitotic divisions to produce undifferentiated progeny to form a syncytium (cyst) of two cells by intercytoplasmic bridge (A_{paired}: A_{pr}) and then of 4, 8, 16, and occasionally 32 cells ($A_{aligned}$: A_{al}) (Yoshida, 2012). Thus A_{undiff} includes A_s, A_{pr} and A_{al} spermatogonia, and the undifferentiated spermatogonia population is heterogeneous (Yoshida, 2012; Kubota and Brinster, 2018), but in primates, this population is made up of two types of A_s spermatogonia referred to as Adark (A_d) and Apale (A_p) (Oatley and Brinster, 2012). Regardless of the species, the A_s spermatogonia are thought to represent or include the SSC population during steadystate conditions, and their number is calculated to be as low as 0.01–0.03% of total adult testis cells (Tegelenbosch and de Rooij, 1993). An undifferentiated spermatogonium of A_{al} divides to transform itself to A1 differentiating spermatogonia, which undergo further five serial mitotic divisions to produce A2, A3, A4, intermediate, and type B differentiating spermatogonia. Type B spermatogonia then differentiate into spermatocytes that undergo meiosis and give rise to haploid spermatids that finally differentiate into spermatozoa (de Rooij DG and Russell, 2000). In recent years, several investigations have been done to determine actual natures of SSCs on morphological, molecular and functional basis (Schulze, 1979; Dettin *et al*., 2003; Phillips *et al*., 2010; Ogawa, 2011; Martin and Seandel, 2013; Wang *et al*., 2019).

However, it is still difficult to clearly identify SSCs because SSCs cannot be precisely identified by a few molecular markers and many of phenotypic characteristics of SSCs overlap with their progeny.

A balance between the self-renewal and differentiation of SSCs in the adult testis is essential to maintain normal spermatogenesis and fertility (Waheeb and Hofmann, 2011). Defects in either the self-renewal or differentiation of SSCs result in the arrest of spermatogenesis that leads to depletion of sperm and therefore infertility (Zhou and Griswold, 2008; Dissanayake, 2018). These two processes are tightly regulated by intrinsic signals in SSCs and extrinsic factors from the surrounding microenvironment, known as the SSC niche (La *et al*., 2018). The concept of stem cell niche was first proposed by Schofield in 1978, to describe a microenvironment that supports stem cells in the mammalian hematopoietic system (Schofield, 1978). Other stem cell niches have now been identified in most tissues of model organisms, including the intestine, skin, brain and testis (Kostereva and Hofmann, 2008). Stem cell niches define as a complex interplay of stimuli among stem cells, neighboring cells, and extracellular matrixes, collectively making up a microenvironment that controls stem cell behaviors, including self-renewal, pluripotency, quiescence and the ability to differentiate into single or multiple lineages (Adams and Scadden 2006; Oatley and Brinster, 2012). The SSC niche mediates extrinsic factors that regulate the self-renewal and differentiation of SSCs and ultimately regulate testicular homeostasis. In this mini-review, the author focuses the recent advances on: (1) identification of spermatogonial stem cells (SSCs); (2) cellular components of the SSC niche; (3) preferential location of the SSC niche in testis in mouse model.

2. Identification of spermatogonial stem cells (SSCs)

Spermatogonial stem cells (SSCs) continuously produced spermatozoa through spermatogenesis (Jan *et al*., 2012). The precise identity of SSCs is still being refined. Studies of well-characterized stem cells reveal why it is difficult to use markers to initially identify unknown stem cells. Stem cells do not simply exhibit constant signaling profiles, but rather these vary depending on the behavior of neighboring cells and their physiological environment (Morrison and Spradling, 2008). The original SSC model, known as the A_s model, proposed that A_s are definitive stem cells and the formation of cysts among their progeny is considered to reflect commitment to differentiation (Huckins, 1971; de Rooij, 1973, 2001; Meistrich and van Beek, 1993; Chan *et al*., 2014). However, in recent years, the A_s model has been challenged and refined by approaches including detailed expression analyses on a set of germ cell marker molecules (reviewd in Phillips *et al*., 2010; Chen and Liu, 2015) and live cell imaging on fluorescent-labeled type A spermatogonia (Kanatsu-Shinohara and Shinohara, 2013).

It is now clear that the A_s population is heterogeneous with a subset of A_s normally functioning as SSCs. It has been recently shown that, in addition to their varying cyst length, A_{undiff} includes A_s, A_{pr} and A_{al} spermatogonia are also characterized by variable levels of gene expression (Phillips *et al*., 2010; Chen and Liu, 2015): GFRα1(GDNF family receptor alpha-1) and NANOS2 (Nanos C2HC-Type Zinc Finger 2) are expressed in a large subset of A_s and A_{pr} that is capable of steady-state self-renewal, contains the great majority of SSC activity and can consider as actual SSCs, while many of A_{al} are $NGN3^+$ (Neurogenin-3: basic helix-loop-helix transcription factor), normally acts as transit-amplifying cells that will eventually undergo differentiation and meiosis. However, expression of GFRα1 and NGN3 exhibits internal heterogeneity in undifferentiated spermatogonia population (Yoshida *et al*., 2004, 2010; Suzuki *et al*., 2009; Zheng *et al*., 2009; Nakagawa *et al*., 2010). Thus, two major populations of A_{undiff} can be distinguished by the expression status of GFRα1 and NGN3. However A_{undiff} in the $NGN3^+$ population still possess the potential to self-renew, *i.e*., capable of switching to a $GFR\alpha1^+$ state and contributing to the stem cell pool (Nakagawa *et al*., 2007, 2010; Sada *et al*., 2009; Suzuki *et al*., 2009; Barroca *et al*., 2009) and can consider as the potential SSCs based on the definition proposed by Potten and Loeffler (1990). They defined potential SSCs as an immediate progeny of actual SSCs that still retain stem cell potential (stemness) while usually undergoing differentiation as follows revealed in the recent studies.

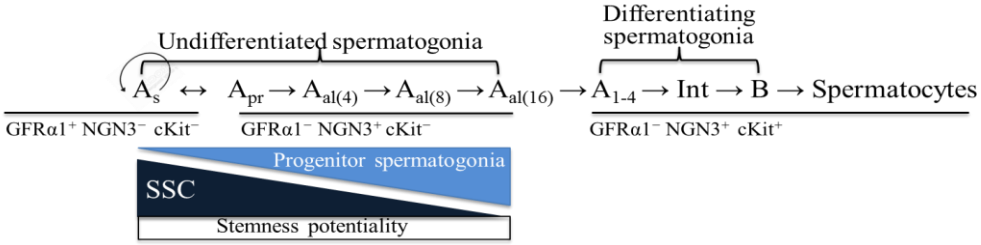

Figure 1. Differential expression of $GFR\alpha1^+$, $NGN3^-$ and cKit genes in stem (SSC), progenitor and differentiating spermatogonia.

Whether SSC division is a symmetric or asymmetric process in mammals remains a topic of debate. Regardless of division pathways, SSCs undergo either self-renewal, to maintain a pool of SSCs, or differentiation, committing to differentiating into spermatogonia and then entering meiosis. Spermatogonia may be basically classified into three categories: GFRα1$^+$ NGN3$^-$ cKit$^-$ (A_{single} spermatogonia, a potential candidate of SSC), GFRα1$^-$ NGN3$^+$ cKit$^-$ (progenitor spermatogonia, they are transit-amplifying cells that will eventually undergo differentiation process but still possess the potential to self-renew), and GFRα1$^-$ NGN3$^+$ cKit$^+$ (differentiating spermatogonia). While stem cell activity is considered to reside in the pool of A_s spermatogonia, the tapered triangle on the below indicates that stem cell activity may extend to Apr and some Aal spermatogonia. The succession of the various types of germ cells during the early stage of mouse spermatogenesis is represented in the image: A_s, A_{single} spermatogonia; A_{pr}, A_{paired} spermatogonia; A_{al}, $A_{aligned}$ spermatogonia; A_{1-4}, types A_1–A_4 spermatogonia; Int, intermediate spermatogonia; B, type B spermatogonia.

A_{undiff} forming cysts are heterogeneous in GFRα1 and NGN3 expression profile except for those in cysts of A_{al-16}, which are essentially NGN3$^+$. A_{undiff} in shorter cysts (mainly $_{Apr}$) and A_s show a higher probability of being GFRα1$^+$ and NGN3$^-$ (Hara *et al*., 2014) whereas A_{undiff} in longer cysts (mainly A_{al}) tend to be GFRα1$^-$ and NGN3$^+$ (Nakagawa et al., 2010) suggesting that A_{undiff} make a transition generally from being GFRα1$^+$ NGN3$^-$ to GFRα1$^-$ NGN^{3+} and then differentiate to cKit$^+$ A1 spermatogonia. Regardless of cell number in cysts, NGN3$^+$ populations (GFRα1$^-$ NGN3$^+$ and GFRα1$^+$ NGN3$^+$ subpopulation) in A_{undiff} including A_s are destined for differentiation indicating that all differentiating spermatogonia, which turn to express c-Kit, a spermatogonial maker for commitment to differentiation (Zhang *et al*., 2011), are derived from NGN3$^+$ cells (Yoshida *et al*., 2004, 2006). Thus, not all A_s behave equivalently as SSCs (Nagano, 2003). Moreover differentiation does not follow a strictly linear process in spermatogenesis. For example, NGN3$^+$ A_{al-4} can be generated either by division of GFRα1$^{+/-}$ NGN^{3+} A_{pr} or by gain of NGN3 expression in GFRα1$^+$ NGN3$^-$ A_{al-4}. Furthermore, A_{pr} and A_{al} are not committed unidirectionally for differentiation but are capable of reverting to A_s by reversion in gene expression or by clone fragmentation in the cysts (Nakagawa *et al*., 2010; Hara *et al*., 2014). By contrast, differentiation potential seems to likely increase and potential for reverting to GFRα^{1+} A_s/SSCs likely decrease correspond to cell numbers in cysts formed by A_{undiff} because differentiation frequencies into A1 spermatogonia are higher in cysts larger in A_{undiff} cell number. GFRα1$^+$ A_s population, which may have the greatest potential to act as SSCs, preferentially maintain steady-state spermatogenesis. However in case of regeneration after tissue damage in the testis where most GFRα1$^+$ A_{undiff} die, NGN3$^+$ spermatogonia including A_s, A_{pr} and A_{al} (nonstem cell undifferentiated spermatogonia) revert to be GFRα1$^+$ SSCs to maintain themselves by self-renewal and continue to produce progeny (Nakagawa *et al*., 2010). Even, the whole mount study suggests cKit-expressing type A1 spermatogonia possess SSC potential (Barroca *et al*., 2009). Overall, A_{undiff} seem to possess a variable level of potential to act as SSCs; GFRα1$^+$ population in A_s has the greatest and NGN3$^+$ population in A_{undiff} has comparatively much lower potential to become SSCs (Figure 1) and their potential is crucial for sperm production capacity in the testis. However, it is still challenging to distinguish SSCs clearly from A_{undiff}.

3. Cellular components of SSC niche

Niche-derived extrinsic stimuli and intrinsically expressed factors regulate stem cell self-renewal and differentiation that provide the foundation for testis homeostasis (Reinwald *et al*., 2016). Unlike the well-defined and distally localized germline stem cell niche in the gonads of other model organisms, such as Drosophila and C. elegans, the mammalian testis contains many potential germline stem cell niches (in the range of several thousand to 40,000 per testis) (Tegelenbosch and de Rooij, 1993; Shinohara *et al*., 2001) that are not well characterized, but are believed to be randomly located along the tubules. In part because the SSC niche lacks a stereotypic location (DeFalco *et al*., 2015), it has proven difficult to define its cellular constituents in mammals.

SSCs reside on the basement membrane of the seminiferous epithelium and contact with Sertoli cells, the only somatic cells forming the framework of the seminiferous epithelium (de Rooij, 2001; Kostereva and Hofmann, 2008; Waheeb and Hofmann, 2011). Peritubular myoid cells, Leydig cells, testicular macrophages, and vascular cells are present in the interstitium and are separated from SSCs by the basement membrane (Smith and Walker, 2014; Potter and DeFalco, 2017; Gofur and Ogawa, 2019; Gofur *et al*., 2020). Thus cell−cell communication not via cell−cell contact but extrinsic factors occurs between SSCs and the interstitial cells. Recently several factors are identified to regulate proliferation and differentiation of SSCs. Glial cell-derived neurotropic factor (GDNF), a member of the transforming growth factor β superfamily, promotes survival and differentiation in a variety of cells (Meng *et al*., 2000). In testis, GDNF is expressed in Sertoli cells and peritubular myoid cells (Tadokoro *et al*., 2002; Chen *et al*., 2016), and its receptors, GFRα1 and RET (GFRα1 is a GPI-linked cell surface receptor for GDNF and mediates activation of the RET receptor tyrosine kinase) are expressed in germ

cells including SSCs (Kubota *et al*., 2004; Fouchécourt *et al*., 2006). Thus GDNF signaling occurs in SSCs and modulates the survival and proliferation (self-renewal) of SSCs (Hofmann, 2008). A high concentration of GDNF promotes proliferation of undifferentiated spermatogonia, and a low concentration of GDNF induces spermatogonial differentiation (Meng *et al*., 2000; Kubota *et al*., 2004; Aponte *et al*., 2008; Wu *et al*., 2009). Not surprisingly, expression of the GDNF receptor, which consists of GFRα1 and RET, on SSCs is also critical for their fate determination. In particular, GFRα1 is high in A_s spermatogonia, which contain SSCs, but the expression decreases in A_{pr} and A_{al} spermatogonia (Nakagawa *et al*., 2010). Basic fibroblast growth factor (bFGF) is secreted by Sertoli cells (Tadokoro *et al*., 2002) and Insulin-like growth factor-1 (IGF-1) is secreted by Leydig cells. They also influence the survival and proliferation of SSCs along with GDNF (Huang *et al*., 2009; Kanatsu-Shinohara and Shinohara, 2013; Muller *et al*., 2017). On the other hand, colony stimulating factor-1 (CSF-1, M-CSF) and retinoic acid (RA) are two key signaling pathways involved in the progression of spermatogenesis. CSF-1 is expressed in Leydig cells, peritubular myoid cells, testicular macrophages and vascular smooth muscle cells (DeFalco *et al*., 2015; Oatley *et al*., 2009) and its receptor is expressed in germ cells including SSCs and the exposure of CSF-1 to SSCs alters the fate decision of self renewal versus differentiation (Oatley *et al*., 2009). Another member of the fibroblast growth factor family, FGF9, has been recently shown to maintain expression of pluripotency-related genes and acts as an inhibitor of meiosis (Barrios *et al*., 2010; Bowles *et al*., 2010). Finally, Kit ligand (KitLG, stem cell factor, SCF), which is also produced by Sertoli cells, induces the differentiation of A_{al} spermatogonia into A1 cells (Pellegrini *et al*., 2008). Testicular macrophages and Leydig cells are thought to produce RA because two essential RA synthesis enzymes, ALDH1A2 and RDH10, are expressed in testicular macrophages and Leydig cells (DeFalco *et al*., 2015). RA stimulates c-Kit expression in spermatogonia and RA and c-Kit together direct the differentiation of spermatogonia (Basuda *et al*., 2015). Vasculature influences SSC dynamics through CSF1 and vascular endothelial growth factor (VEGF) (Potter and DeFalco, 2017). Moreover, these extrinsic signals will modulate SSC intrinsic signals such as kinases, second messengers and transcription factors to ensure homeostasis (Kostereva and Hofmann, 2008). Studies demonstrate that, Sertoli cells and interstitial cells including Leydig cells, testicular macrophages, peritubular myoid cells and vascular smooth muscle cells provide several essential extrinsic factors influencing SSC fate decision. Thus these cells along with SSCs form the special microenvironment that called SSC niche (Figure 2) crucial for SSC self-renewal and differentiation indicating that these cells are components of SSC niche.

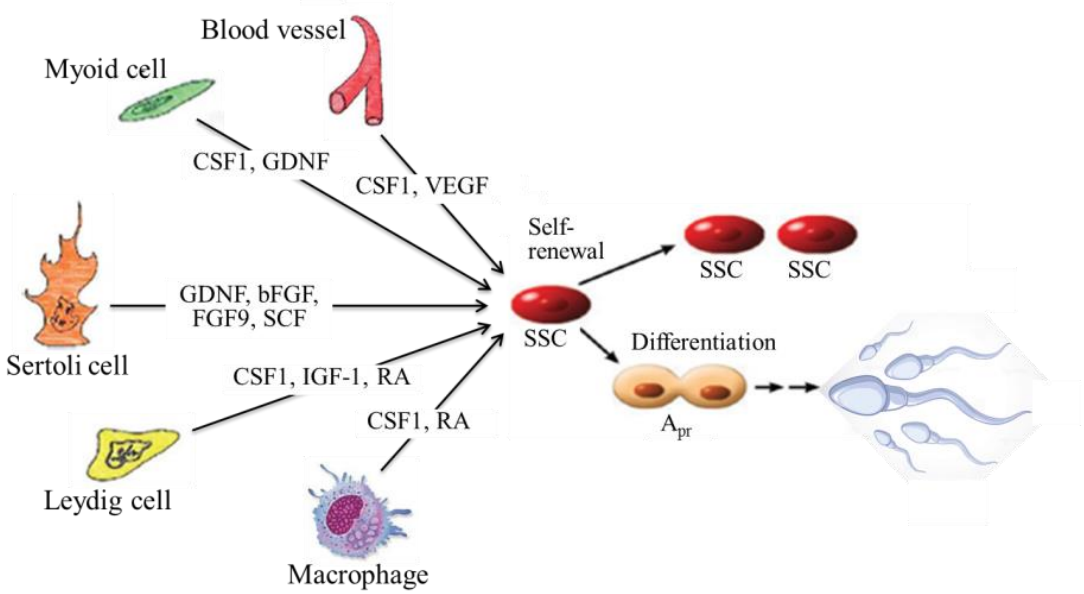

Figure 2. Cellular components of SSC niche. Sertoli cell and intersticial cells including Leydig cell, myoid cell, macrophage and smooth muscle cells of interstial blood vessels secrete several extrinsic factors influencing the SSCs fate decision (either self-renewal or differentiation, on demand). Thus these cells along with SSCs form the special microenvironment, the SSC niche.

4. Preferential location of SSC niche

Niches have a defined anatomical location (Schofield, 1978; Jones and Wagers, 2008). SSCs are functionally linked to their niche or special microenvironment that influences their behaviors on self-renewal and differentiation (Kostereva and Hofmann, 2008). A_{undiff} including SSCs are scattered throughout seminiferous tubules in the testis and thus the SSC niche seems to be also scattered (Hara *et al*., 2014; Ikami *et al*., 2015). It is important to determine where the SSC niche locates in the testis because the nature of SSC niche is directly related to the nature of SSCs (Kostereva and Hofmann, 2008). However, no specific substructures have been identified yet for the SSC niche.

SSCs cannot be clearly identified in the tissue of the testis yet but a subset of A_{undiff} constitutes a pool of SSCs (Yoshida, 2012; Kubota and Brinster, 2018). Thus many studies have examined the preferential location of A_{undiff} in the testis in order to identify the SSC niche and SSCs as a consequence. $GFR\alpha1^+$ cells comprise 30–40% of the entire undifferentiated spermatogonia (Nakagawa *et al*., 2010). Although $GFR\alpha1^+$ A_{undiff} (a most likely candidate for SSCs) are found over the basal compartment seminiferous tubules, immunostaining studies demonstrated that they show biased localization to the area adjacent to the interstitium that always coincide blood vessels (Chiarini-Garcia *et al*., 2001, 2003; Compos-Junior *et al*., 2012; de Rooij, 2015). On differentiation, they migrate from this region to spread over the basal compartment, which has been implicated by careful investigation of testis sections and observed directly by intravital live-imaging studies (Chiarini-Garcia *et al*., 2003; Hara *et al*., 2014; Yoshida *et al*., 2007). $NGN3^+$ spermatogonia, which are predominantly composed of A_{undiff} but not the entire, and give rise to all types of spermatogonia in the mature testis (Yoshida *et al*., 2004, 2006). The GFP-labeled $NGN3^+$ spermatogonia showed variable distribution patterns corresponding to the stages of seminiferous epithelia (spermatogenic stages): they preferentially located in the region of seminiferous tubules adjacent to blood vessels at stages IV to VI, and their vascular preference became weaker at stages VII and VIII, when the A_{al} to A1 transition occurs (Chiarini-Garcia *et al*., 2003; Yoshida *et al*., 2007). Time-lapse imaging and three dimensional reconstruction, and also in allograft of the seminiferous tubule fragment show a clear spatial correlation between A_{undiff} and blood vessels (Yoshida *et al*., 2007): A_{undiff} preferentially located in the region adjacent to the bifurcation of blood vessels in the normal testis as well as in the graft of seminiferous tubule fragments. Thus, not only $GFR\alpha1^+$ A_{undiff} (a most likely candidate for SSCs) but also $NGN3^+$ A_{undiff} preferentially locate in the region of seminiferous tubules adjacent to blood vessels especially at the branching points of blood vessels, and this may indicate the SSC niche forms in close association with blood vessels.

5. Conclusions

According to recent advances on identification of SSCs and their niche, the following can be concluded: (1) A_{undiff} seem to possess variable levels of stem cell potential to act as SSCs; $GFR\alpha1^+$ population in A_s has the greatest potential to act as SSCs (can consider as actual SSCs) whereas $NGN3^+$ population in A_{undiff} has comparatively much lower potential to act as SSCs (can consider as potential SSCs). (2) Sertoli cells and interstitial cells including Leydig cells, testicular macrophages, peritubular myoid cells and vascular smooth muscle cells together with SSCs form a special microenvironment called “SSC niche” by secreting several essential extrinsic factors crucial for SSC self-renewal and differentiation suggesting that these cells are the components of SSC niche. (3) $GFR\alpha1^+$ A_{undiff}, a most likely candidate for SSCs, preferentially locate in the region of seminiferous tubules adjacent to blood vessels especially at the branching points, indicates that the SSC niche forms in close association with blood vessels.

Conflict of interest

None to declare.

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