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学位（専攻分野） 博士（理学）

学位記番号 総研大甲第 1430 号

学位授与の日付 平成 23 年 3 月 24 日

学位授与の要件 生命科学研究科 遺伝学専攻  
学位規則第 6 条第 1 項該当

学位論文題目 The RNA-binding protein NANOS2 is required to  
maintain murine spermatogonial stem cells

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## 論文内容の要旨

Spermatogonial stem cells represent a stem cell population in adult testes, and their biological activities provide a base of continuous production of spermatozoa. The stem cell function resides in undifferentiated spermatogonia, consisting of types  $A_{\text{single}}$ ,  $A_{\text{paired}}$  and  $A_{\text{aligned}}$ . These cells transform into type  $A_1$  differentiating spermatogonia, which subsequently undergo six mitotic and two meiotic divisions to form haploid spermatozoa within 35 days. The entire developmental process from spermatogonia to spermatozoa occurs within seminiferous tubules of testes. In the tubules, all types of spermatogonia are located on the basement membrane, and the subsequent differentiating cell types are arranged in a sequential order towards the lumen. The spermatogenesis is also supported by close interaction of the germ cells with somatic Sertoli cells.

Currently, several factors have been implicated in the regulation of spermatogonial stem cells. For example, a growth factor GDNF (glial cell-line derived neurotrophic factor) is secreted by Sertoli cells and acts as one of the major niche signals for spermatogonial stem cells. A transcription factor PLZF (promyelocytic leukemia zinc finger) is needed in a cell autonomous fashion for the maintenance of the spermatogonial stem cells, since male mice lacking *Plzf* expression exhibit progressive germ cell loss and testis atrophy with age, causing infertility. However, the previous loss of function studies had some limitations in terms of understanding the mechanisms by which stem cells are lost upon the gene deletion, as it could be caused by cell death, a defective proliferation, a premature differentiation, or other mechanisms.

A precise identification of stem cells is another important issue in stem cell biology. According to “ $A_{\text{single}}$  model”,  $A_{\text{single}}$  spermatogonia represent spermatogonial stem cells: this type is recognized as single cells without any intercellular connection

with others, whereas  $A_{\text{paired}}$  and  $A_{\text{aligned}}$  spermatogonia are connected by intercellular cytoplasmic bridges and committed to differentiation. However, the stem cell activity of the  $A_{\text{single}}$  spermatogonia has not been tested due to a lack of their specific molecular markers.

NANOS, a zinc-finger RNA-binding protein, has been proposed as an evolutionarily conserved factor for germline cell function. Among three NANOS homologs in mice, NANOS2 is essential for the male-type differentiation of embryonic germ cells. In adult testes, NANOS2 is predominantly expressed in the  $A_{\text{single}}$  and  $A_{\text{paired}}$  spermatogonia, suggesting that NANOS2 may also be involved in the spermatogonial stem cell function. However, the postnatal function of NANOS2 has not been studied, because the majority of *Nanos2*-null male germ cells undergo apoptosis before birth.

In the first part of my doctoral thesis, I report my findings indicating that the RNA-binding protein NANOS2 is a key regulator for the maintenance of spermatogonial stem cells. First, by lineage-tracing analyses, I revealed that undifferentiated spermatogonia expressing *Nanos2* retained abilities to self-renew and generate the entire spermatogenic cell lineage. Hence, such spermatogonia can be referred to as spermatogonial stem cells. Next, I addressed if NANOS2 plays a role in these stem cells by using a conditional gene knockout system. I showed that conditional disruption of postnatal *Nanos2* led to depletion of spermatogonial stem cell reserves and resulted in the complete depletion of germ cells within a few cycles of spermatogenesis. These results indicate that NANOS2 is expressed in spermatogonial stem cells and is required for the maintenance of these cells.

The current view of the regulation of stem cell state or “stemness” depicts following cellular processes: (1) cell proliferation to expand stem cell population; (2)

maintenance of the undifferentiated state; and (3) cell death/survival. NANOS2 should control these events for maintaining the proper state of spermatogonial stem cells. To further determine the role of NANOS2 in the stem cell maintenance, I generated transgenic mice, which allowed continuous expression of NANOS2 in germ cell lineages. I found that mouse testes in which *Nanos2* had been overexpressed accumulated spermatogonia with undifferentiated, stem cell-like properties. Furthermore, these *Nanos2*-overexpressing cells showed lower proliferation rates and similar levels of apoptosis compared to the control spermatogonia. These results indicate that NANOS2 maintains spermatogonial stem cells by suppressing proliferation and differentiation of these cells.

Studies in diverse stem cell systems indicate that the stem cell regulations are dependent on not only stem cell-intrinsic factors but also extrinsic signals from the microenvironment known as niche signals. However, it remains unclear how extrinsic signals and intrinsic stem cell factors intersect in stem cells to control their cellular state. During murine spermatogenesis, GDNF signals from Sertoli cells and germ cell-intrinsic factor NANOS2 represent key regulators for the maintenance of spermatogonial stem cells. In the second part of my thesis, I examined the possible genetic interaction between NANOS2 and GDNF signal transduction pathway.

First, I conducted conditional knockout of *Gfra1*, a receptor for GDNF, to decipher roles of the GDNF signaling during adult spermatogenesis. The absence of *Gfra1* resulted in rapid loss of spermatogonial stem cells, which might result from deficits in proliferation and/or in the maintenance of undifferentiated state. Next, I asked whether the overexpression of *Nanos2* could negate the stem cell loss phenotype caused by the *Gfra1*-deletion. I found that overexpressed *Nanos2* could not support

*Gfra1*-mutant stem cells permanently, because proliferation of spermatogonial stem cells was severely impaired by the lack of GDNF signaling. This result indicates that stem cell proliferation is highly dependent on the GDNF-stimulated pathway.

On the other hand, NANOS2 did prevent precocious differentiation of spermatogonial stem cells caused by the *Gfra1*-deficiency, indicating that the stem cell differentiation could be suppressed solely by NANOS2 independent of GDNF signaling. The GDNF-independent NANOS2 function was further assessed by the ectopic expression of *Nanos2* in GFRA1-negative spermatogonia. The results support my idea that NANOS2 is capable of maintaining undifferentiated state of spermatogonial stem cells in the absence of GDNF-signaling pathway. Taken together, I suggest that the stem cell-intrinsic factor NANOS2 and extrinsic GDNF signals coordinately maintain spermatogonial stem cells by acting through different cellular and/or molecular mechanisms. My doctoral studies thus offer a novel insight into understanding how stem cells are maintained during murine spermatogenesis.

## 博士論文の審査結果の要旨

マウスの精子形成は精原幹細胞 (SSC) によって維持されているが、どの精原細胞が幹細胞特性を持つのか、さらにその幹細胞特性がどのように制御されているのかはまだ不明である。佐田亜衣子さんの所属研究室では、RNA 結合タンパク NANOS2 が未分化型精原細胞の中でも特に初期型でのみ発現することを見つけていた。しかしながら、*Nanos2* は始原生殖細胞から発現しており、単純な遺伝子ノックアウトでは生殖細胞そのものが欠失することから、精巣での機能解析は困難であった。そこで、佐田さんはコンディショナルノックアウトマウスやトランスジェニックマウスなどの技術を用いて以下の研究を行った。

まず、*Nanos2* が発現した細胞で *lacZ* が恒常的に発現するトランスジェニックマウスを作製して、その細胞の系譜を追跡した。その結果、*Nanos2* 発現細胞が自己再生するとともに分化型の精子形成細胞を生み出す幹細胞として機能することが明らかとなった。さらに、*Nanos2* をコンディショナルノックアウトすることで初期型精原細胞がなくなること、その過剰発現により初期型精原細胞が蓄積することを認めた。これらの結果は NANOS2 が SSC の幹細胞特性を維持するために不可欠な因子であることを示すものである。

次に、SSC を維持する精巣内微小環境、いわゆる幹細胞ニッチの主要因子と考えられている GDNF (glial cell-line derived neurotrophic factor) に着目し、NANOS2 との関連性について調べた。コンディショナルに *Nanos2* が過剰発現し GDNF 受容体をコードする *Gfra1* が欠損するトランスジェニックマウスを作製して、その影響を調べたところ、*Nanos2* の過剰発現は *Gfra1* 欠損による初期型精原細胞の消失を部分的に救済したが、完全に救済することはできなかった。GDNF は SSC の分化抑制と増殖の促進という 2 つの作用により SSC を維持することが知られている。一方、*Nanos2* の過剰発現は SSC の分化抑制という機能については救済するが、SSC の増殖については GDNF の作用とは逆にむしろ抑制的に働くことが示された。これらの結果は、GFRA1 の下流には NANOS2 とは独立に SSC の幹細胞特性を維持する経路もあることを示唆している。

佐田さんは緻密なコンディショナルノックアウト技術を駆使して、NANOS2 が SSC の維持に不可欠な因子であることを示しており、その成果は幹細胞特性維持の分子機構の一端を明らかにしたものとして意義は大きい。さらに、NANOS2 が GDNF-GFRA1 シグナルとは独立して SSC で機能する可能性を示し、今後の進展が待たれる興味深い成果をあげている。以上の理由から、佐田さんの博士論文は博士号授与の要件を満たすと審査員全員一致で判断した。