博士論文（要約）

Study of the Sertoli Valve Epithelia in the Terminal Segment of Mouse Seminiferous Tubules

（マウス精細管基部のセルトリバルブ上皮に関する研究）

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獣医学専攻　博士課程　猪村（貴志）　かさね

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This doctorate thesis is anticipated to be published as a jointly authored paper in a scholarly journal, and as the permission of all collaborating authors has not been granted, it cannot be published online. The paper is scheduled to be published within 5 years.

論文の内容の要旨

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論文題目

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In adult mammalian testes, spermatozoa are produced through almost all their life, and this is because males have spermatogonial stem cells (SSCs) inside testes, and SSCs self-renew or differentiate in a well-balanced manner. SSCs are settled in seminiferous tubules with nursing cells called Sertoli cells (SCs) and SCs support the SSCs. SCs supply secreting factors and regulate the balance of self-renewing and differentiation of undifferentiated spermatogonia including SSCs. A major niche factor for SSCs' self-renewing is glial cell line-derived neurotrophic factor (GDNF). Undifferentiated spermatogonia including SSCs express GDNF family receptor alpha 1 (GFRA1). On the other hand, major SSCs' differentiating factor is retinoic acid (RA).

Undifferentiated spermatogonia differentiate into differentiated spermatogonia and after that, they differentiate into spermatocytes and meiosis occurs. By meiosis, haploid cells, called spermatids, are produced. Spermatids undergo the morphological change and develop into matured spermatids. Finally, they are released into the lumen of seminiferous tubules as spermatozoa and transferred into the rete testis (RT) by the luminal fluid flow. This series of events is called spermatogenesis. Spermatogenesis is

precisely coordinated in time and space and occurs with periodical cycle called seminiferous epithelial cycle. In mice, it has been divided into 12 stages. These cyclical differentiations might be explained by periodical GDNF secretion and RA secretion. The stages highly expressing GDNF and RA occur alternatively like a seesaw.

The above periodical differentiation is the story inside convoluted seminiferous tubules (ST), which account for a large portion of seminiferous tubules. In the terminal segment of seminiferous tubules, where they are connected to rete testis, a gradual depletion of germ cells occurs and finally tubules are lined only by SCs and a few spermatogonia. This region is known as straight seminiferous tubule, transitional zone or transition region, and this region is seen in many species including human. The SCs in this region form 'plug' or 'valve' -like structure and are considered to regulate the luminal fluid flow of seminiferous tubules. In our laboratory, we call this structure "Sertoli valve (SV)" and our previous data on hamsters show that SV epithelium constitutively expresses GDNF and supports the stable proliferation and selective maintenance of undifferentiated spermatogonia. The SCs in SV region support GFRA1-positive undifferentiated spermatogonia, while the region lacks KIT-positive differentiated spermatogonia and further differentiated spermatogenic cells. These data suggest that in SV region, balance between self-renewal and differentiation of SSCs is inclined more to self-renewal, so that periodical cycle is missing.

SCs proliferate from fetal stage to neonatal stage, however for a long time, it was considered that proliferation of SCs is not observed in adult stage in most of mammals. Our previous data show that SCs in SV region of hamsters are still capable of proliferation even in adult stage. Moreover, the evidences of proliferating adult SCs in SV region were also found in normal rat testes and cultured mouse SCs.

As stated above, SV region shows interesting feature, so that many researchers hope to know the cellular or molecular characteristics. In this study, I did the histological observation, developmental engineering analysis, and transciptomic analyses to clarify the development and molecular basis of SV structure. For the histological observation, I focused on the Akt signaling and performed the immunohistochemistry using anti-phospho-Akt (active form of Akt) antibody. In the developmental engineering analysis, I used Amh-Treck transgenic mice to deplete the SV structure and after that, I reconstruct the SV structure by SCs of convoluted seminiferous tubules. Finally, I performed the microarray and detected the SV specifically highly expressed genes.

In Chapter 1, I analyzed the activation of Akt signaling in SCs using immunostaining of anti-p-Akt antibody. In ST region, Akt signaling was seminiferous epithelial cycle-dependently activated, while in SV region, Akt signaling was constitutively activated. This pattern of Akt activation in SV region was not observed in 1-week-old neonatal mouse testes, but from 2 to 4-week-old, this pattern was observed and the valve-like structure was constructed. Transplanting the immature 1-week-old mouse SCs of ST region into SCs-depleted Amh-Treck Tg mouse testes revealed that SCs of ST can act as SCs of SV such as supporting few spermatogenic cells, activation of Akt signaling, and construction of valve-like structure positive for ace-Tub, when they are settled in SV region. These data suggest that Akt signaling is region-specifically constitutively activated after birth, and the construction of valve-like structure non-cell autonomously occurs dependent on information of the place where SCs are settled. I cannot understand the activation of Akt signaling in SV is the result or cause of the construction of valve-like structure from these data, but activation of Akt signaling in SV region might play an important role in constructing SV structure.

In Chapter 2, I show that *Cyp26a1*, the RA-metabolizing enzyme, was highly expressed in SCs of SV region of W/W^v mice (lack spermatogenic cells due to a germ cell-autonomous defect) compared to RT and ST regions by microarray, quantitative PCR analysis and *in situ* hybridization. In contrast, *Aldh1a2*, the RA-synthesizing enzyme, was highly expressed in ST region of wild-type mice, which include spermatogenic cells, compared to RT, SV, and ST regions of W/W^v mice. RA is the differentiation factor of spermatogenic cells. The data that *Cyp26a1* is highly expressed in SV region suggest that RA degradation is constitutively occurring in SV region, so that SV region is missing seminiferous epithelial cycle, and balance between self-renewal and differentiation of SSCs is inclined more to self-renewal. This is consistent with the previous data that SV epithelia constitutively express GDNF and support the stable proliferation and selective maintenance of undifferentiated spermatogonia. RA degradation in SV region may play an important role in regulating the differentiation of spermatogenic cells in SV region. I also show that *Fgf9* is highly expressed in RT region, and exogenous FGF9 can activate the Akt signaling in SCs even in ST region.

In the present study, I show the constitutive activation of Akt signaling in SV region, and the activation of Akt signaling and formation of valve-like structure non-cell autonomously occurred. Based on these findings, I proposed the hypothesis that RT secretes FGF9 and SCs that received FGF9 will be activated of Akt signaling, and then the SCs show the feature of SV structure. In addition, I cannot forget about the seminiferous epithelial cycle-dependent activation of Akt signaling in wild-type mouse ST region. Clarifying the factors of activating Akt signal in STs and the function of cyclical activation of Akt signaling is required. This cyclical activation of Akt signaling in ST region might have a relation with constitutive activation of Akt signaling in SV region. In the present study, I show that *Cyp26a1* is highly expressed in SV region and that data suggest that RA degradation is constitutively occurring in SV region, and balance between self-renewal and differentiation of SSCs is inclined more to self-renewal in SV region. I can hypothesize that this situation is produced by constitutive activation of Akt signaling. If I apply this hypothesis to ST region, cyclical activation of Akt signaling in ST region may produce the cyclical balance of self-renewing and differentiation of SSCs.

RA signaling plays crucial roles during vertebrate development. There is a concept of RA-FGF antagonism and it is applied to several mechanisms of development. In the chick embryo, FGF signaling has been shown to antagonize the RA gradient and to maintain the undifferentiated state of cells in the posterior part of the embryo throughout somitogenesis. On the research of mouse somitogenesis, conditional deletion in the mesoderm of *Fgfr1*, the only FGF receptor expressed in the mouse paraxial mesoderm, resulted in the absence of the RA-degrading enzyme CYP26 in the posterior part of the embryo. In addition, on the research of mouse limb bud development, culturing of wild-type mouse forelimb bud in the presence of an inhibitor of the FGFR tyrosin kinase (SU5402) resulted in almost complete loss of *Cyp26b1* expression, and conversely, the implantation of FGF4-loaded beads into *Shh*-deficient mouse limb buds, of which the expression of *Cyp26b1* was reduced in the distal mesenchyme and RA activity was increased, resulted in striking restoration of *Cyp26b1* expression. Applying these findings to the present study, I can consider that FGF9 signaling in SV epithelia might activate the expression of *Cyp26a1* in SV epithelia.

General Introduction

A life starts from one zygote. The zygote is formed by fertilization of two gametes, oocyte and spermatozoon. The gametes are haploid cells that contain half number of chromosomes compared to somatic cells. The process for obtaining haploid cells involves the meiotic division inside the gonads.

As noted above, there are two kinds of gametes, oocyte and spermatozoon. In mammalian gonads, oocytes are produced in XX female ovaries, and spermatozoa are produced in XY male testes. Oocytes and spermatozoa are both derived from primordial germ cells (PGCs), but PGCs go through the different processes inside XX ovaries and XY testes. In mammalian XX ovaries, all oocytes, which are differentiated from PGCs, undergo first meiotic division while fetal stage. On the other hand, in mammalian fetal XY testes, PGCs differentiate into spermatogonia and still maintain the pluripotency (Feng *et al.* 2014). The main factor for making this difference is retinoic acid (RA), a derivative of vitamin A. In fetal XX ovaries, RA induces meiosis in fetal stage (Li & Clagett-Dame 2009, Bowles *et al.* 2016), while in fetal XY testes, RA is degraded by RA-metabolizing enzyme, Cytochrome P450, family 26, subfamily b, polypeptide 1 (CYP26B1) (Bowles *et al.* 2006, Koubova *et al.* 2006, MacLean *et al.* 2007).

In adult mammalian testes, spermatozoa are produced through almost all their life, and this is because males have spermatogonial stem cells (SSCs) inside testes, and SSCs self-renew or differentiate in a well-balanced manner (de Rooij 2017, Lord & Oatley 2017). SSCs are settled in seminiferous tubules with nursing cells called Sertoli cells (SCs) and SCs support the SSCs. SCs supply secreting factors and regulate the balance of self-renewing and differentiation of undifferentiated spermatogonia including SSCs

(Franca *et al.* 2016). Outside of seminiferous tubules, there are peritubular cells (myoid cells), interstitial cells (Leydig cells), vasculatures and so on, and they also regulate the fate of undifferentiated spermatogonia (Yoshida *et al.* 2007, Chen *et al.* 2016, Potter & DeFalco 2017). A major niche factor for SSCs' self-renewing is glial cell line-derived neurotrophic factor (GDNF) (Meng *et al.* 2000). Undifferentiated spermatogonia including SSCs express GDNF family receptor alpha 1 (GFRA1) (Grasso *et al.* 2012, Hara *et al.* 2014). On the other hand, major SSCs' differentiating factor is RA (van Pelt & de Rooij 1990, Hogarth & Griswold 2010, Endo *et al.* 2015).

Undifferentiated spermatogonia differentiate into differentiated spermatogonia and after that, they differentiate into spermatocytes and meiosis occurs. By meiosis, haploid cells, called spermatids, are produced. Spermatids undergo the morphological change (elongating, etc.) and develop into matured spermatids. Finally, they are released into the lumen of seminiferous tubules as spermatozoa (this event is called spermiation) and transferred into the rete testis by the luminal fluid flow (Russell *et al.* 1990). This series of events is called spermatogenesis. Spermatogenesis require many kinds of molecules including RA, and previously I revealed that a deubiquitinase USP9X (ubiquitin-specific peptidase 9, X chromosome) is expressed in spermatogonia and is essential for proper spermatogenesis, showing the defective spermatogenesis with reduced numbers of spermatocytes and subsequent failure of spermiation in *Usp9x* conditional knockout mouse testes (Kishi *et al.* 2017). Moreover, spermatogenesis is precisely coordinated in time and space and occurs with periodical cycle called seminiferous epithelial cycle. In mice, it has been divided into 12 stages (Oakberg

1956a, Oakberg 1956b) (Also see Fig. 0-1). These cyclical differentiations might be explained by periodical GDNF secretion (Johnston *et al.* 2011, Sato *et al.* 2011, Grasso *et al.* 2012) and RA secretion (Sugimoto *et al.* 2012, Hogarth *et al.* 2015a, Griswold 2016, Endo *et al.* 2017). The stages highly expressing GDNF and RA occur alternatively like a seesaw.

The above periodical differentiation is the story inside convoluted seminiferous tubules, which account for a large portion of seminiferous tubules. In the terminal segment of seminiferous tubules, where they are connected to rete testis, a gradual depletion of germ cells occurs and finally tubules are lined only by SCs and a few spermatogonia (Fig.0-2). This region is known as straight seminiferous tubule, transitional zone or transition region, and this region is seen in many species including human (Roosen-Runge 1961, Dym 1974, Fawcett & Dym 1974, Osman 1978, Nykanen 1979, Osman 1979, Lindner 1982, Ezeasor 1986, Hermo & Dworkin 1988). The SCs in this region form ‘plug’ or ‘valve’ -like structure and are considered to regulate the luminal fluid flow of seminiferous tubules (Johnson *et al.* 1970, Russell & Griswold 1993). In our laboratory, we call this structure “Sertoli valve (SV)” and our previous data on hamsters (Aiyama *et al.* 2015) show that SV epithelium constitutively expresses GDNF and supports the stable proliferation and selective maintenance of undifferentiated spermatogonia. The SCs in SV region support GFRA1-positive undifferentiated spermatogonia, while the region lacks KIT-positive differentiated spermatogonia and further differentiated spermatogenic cells. These data suggest that in SV region, balance between self-renewal and differentiation of SSCs is inclined more to

self-renewal, so that periodical cycle is missing.

SCs proliferate from fetal stage to neonatal stage, however for a long time, it was considered that proliferation of SCs is not observed in adult stage in most of mammals (Sharpe *et al.* 2003). Our previous data show that SCs in SV region of hamsters are still capable of proliferation even in adult stage (Aiyama *et al.* 2015). Moreover, the evidences of proliferating adult SCs in SV region were also found in normal rat testes (Figueiredo *et al.* 2016) and cultured mouse SCs (Kulibin & Malolina 2016).

As stated above, SV region shows interesting feature, so that many researchers hope to know the cellular or molecular characteristics. In this study, I did the histological observation, developmental engineering analysis, and transciptomic analyses to clarify the development and molecular basis of SV structure. For the histological observation, I focused on the Akt signaling and performed the immunohistochemistry using anti-phospho-Akt (active form of Akt) antibody. In the developmental engineering analysis, I used Amh-Treck transgenic mice (Shinomura *et al.* 2014) to deplete the SV structure and after that I reconstruct the SV structure by SCs of convoluted seminiferous tubules. Finally, I performed the microarray and detected the SV specifically highly expressed genes.

A Although a figure was reproduced here from "Histological and histopathological evaluation of the testis" (Russell et al. 1990), as permission for online publication has not been granted by the copyright holder, it cannot be published here.

B

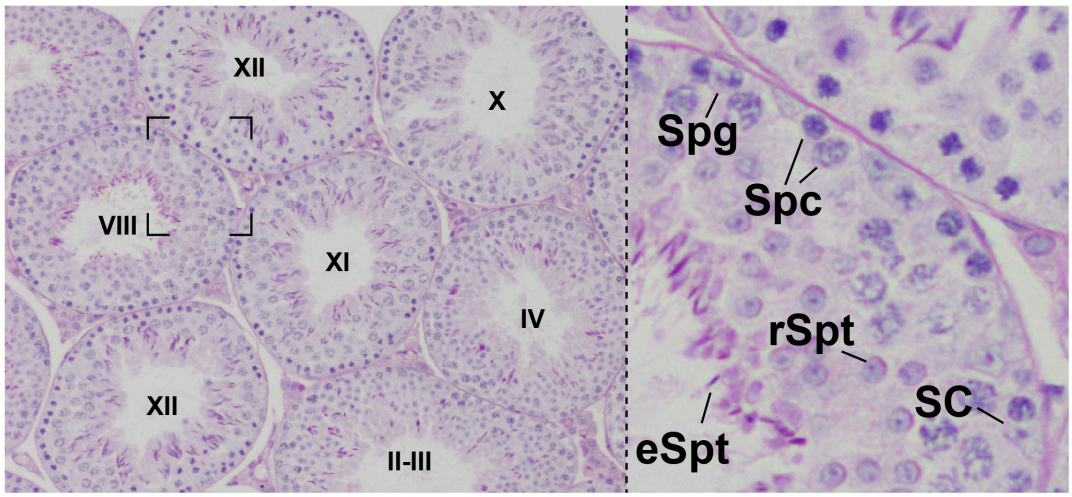

Fig. 0-1

Fig. 0-1. Seminiferous epithelial cyle

(A) Figure from "Histological and histopathological evaluation of the testis" (Russell *et al.* 1990). A map of the seminiferous epithelial cycle of mouse showing catalogued phases of germ cell development. m, mitosis or meiosis of the cells; In, intermediate spermatogonia; B, B spermatogonia; Pl, Preleptotene spermatocyte; L, Leptotene spermatocyte; Z, Zygotene spermatocyte; P, Pacytene spermatocyte; D, Diplotene spermatocyte; 2°, secondary spermatocyte; Arabic numerals, steps of spermatids. **(B)** Transverse section of convoluted seminiferous tubules. Roman numerals indicate the seminiferous epithelial cycle stage of each tubule. Spermatogenic cells in the tubules synchronously develop and show cyclical differentiation, but the adjacent seminiferous tubules do not always show the same cycle stage and they are not synchronously regulated because inside tubules, development of spermatogenic cells occurs as wave along the long axis of tubules. Right panel is higher magnification indicated by broken rectangle in left panel. SC, Sertoli cell; Spg, spermatogonium; Spc, spermatocyte; rSpt, round spermatid; eSpt, elongated spermatid.

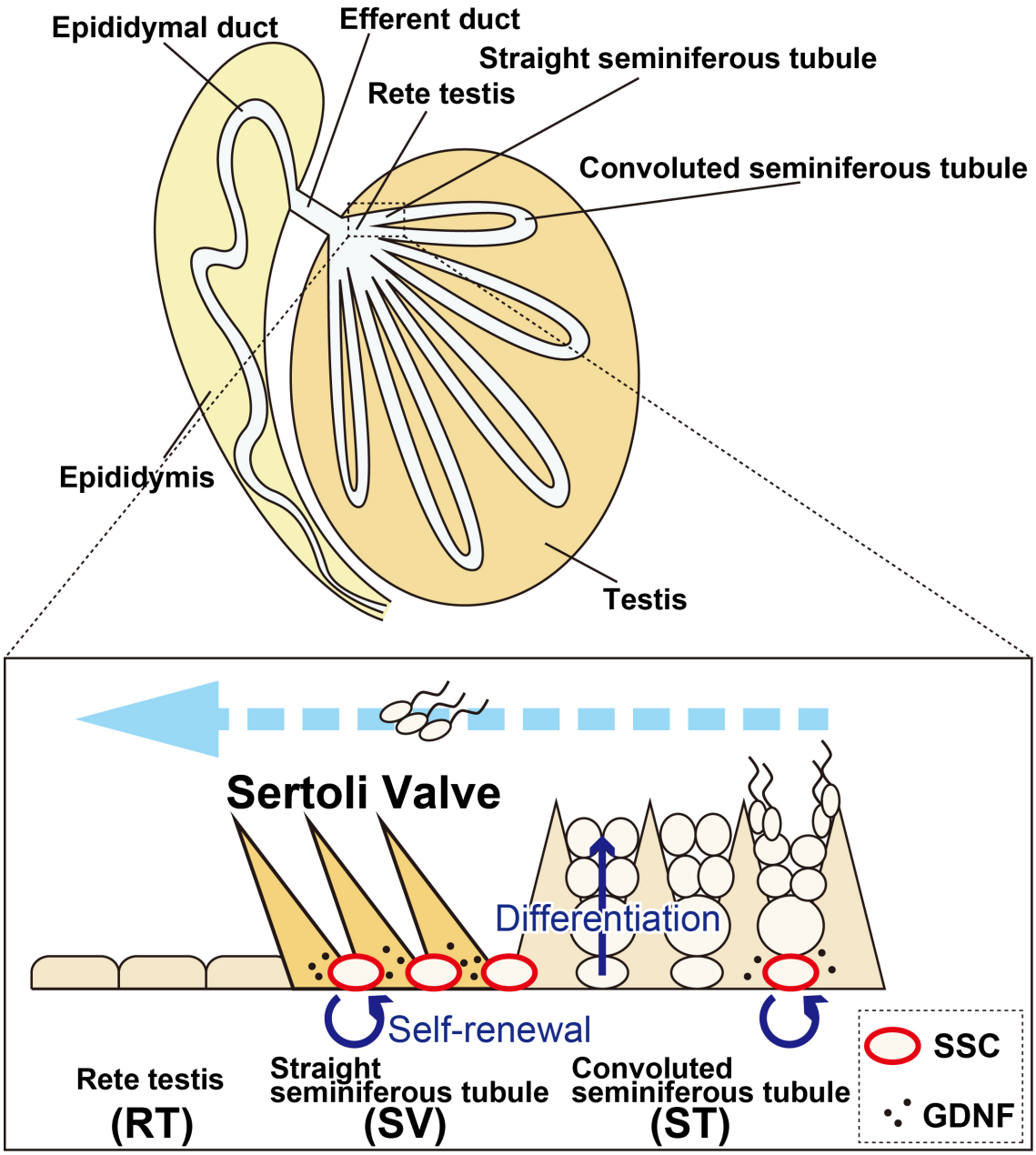
Epididymal duct
Efferent duct
Straight seminiferous tubule
Rete testis
Convoluted seminiferous tubule
Epididymis
Testis
Sertoli Valve
Differentiation
Self-renewal
Rete testis
(RT)
Straight
seminiferous tubule
(SV)
Convoluted
seminiferous tubule
(ST)
SSC
GDNF

Fig. 0-2

Fig. 0-2. Scheme of structures of testis and epididymis

Seminiferous tubules are in the testis, and interstitial cells surround the tubules. Convoluted seminiferous tubules account for a large portion of seminiferous tubules and inside the convoluted seminiferous tubules, spermatogenic cells self-renew and differentiate in a well-balanced manner. In the terminal segment of seminiferous tubules, where they are connected to rete testis, a gradual depletion of germ cells occurs and finally tubules are lined only by SCs and a few spermatogonia.

Chapter 1

Development of Sertoli valve epithelia

As the contents of this chapter are anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 5 years.

Chapter 2

Molecular basis of Sertoli valve epithelia

As the contents of this chapter are anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 5 years.

General Discussion

In Chapter 1, I analyzed the activation of Akt signaling, and in ST region, Akt signaling was seminiferous epithelial cycle-dependently activated, while in SV region, Akt signaling was constitutively activated. This pattern of Akt activation in SV region was not observed in 1-week-old neonatal mouse testes, but from 2 to 4-week-old, it was observed and the valve-like structure was constructed. Transplanting the immature 1-week-old mouse SCs of ST region into SCs-depleted Amh-Treck Tg mouse testes revealed that SCs of ST can be activated of Akt signaling, and construct the valve-like structure positive for ace-Tub as SCs of SV when they are settled in SV region. These data suggest that Akt signaling is region-specifically constitutively activated after birth, and the construction of valve-like structure non-cell autonomously occurs dependent on information of the place where SCs are settled.

In Chapter 2, according to the hypothesis from Chapter 1, I compared the transcription of RT, SV, and ST regions of W/W^v mice. Microarray analysis offered me the 326 SV specifically highly expressed genes, and the top of them was *Cyp26a1*, which encodes the RA-metabolizing enzyme. I confirmed that *Cyp26a1* was highly expressed in SCs of SV region of W/W^v mice compared to RT and ST regions by quantitative PCR analysis and *in situ* hybridization. RA is the differentiation factor of spermatogenic cells and induces the key transitions of spermatogenesis, and periodical secretion of RA enables the cyclical differentiation of spermatogenic cells in convoluted seminiferous tubules (Sugimoto *et al.* 2012, Hogarth *et al.* 2015a, Griswold 2016, Endo *et al.* 2017). The data that *Cyp26a1* is highly expressed in SV region suggest that RA degradation is constitutively occurring in SV region, so that SV region is missing

seminiferous epithelial cycle, and balance between self-renewal and differentiation of SSCs is inclined more to self-renewal. This is consistent with the previous data that SV epithelia constitutively express GDNF and support the stable proliferation and selective maintenance of undifferentiated spermatogonia (Aiyama *et al.* 2015). RA degradation in SV region may play an important role in regulating the differentiation of spermatogenic cells in SV region.

I also show that *Fgf9* is highly expressed in RT region, and exogenous FGF9 can activate the Akt signaling in SCs even in convoluted seminiferous tubules. In Chapter 1, I show the constitutive activation of Akt signaling in SV region, and the activation of Akt signaling and formation of valve-like structure non-cell autonomously occurred. Based on these findings, I proposed the hypothesis that RT secretes FGF9 and SCs that received FGF9 will be activated of Akt signaling, and then the SCs show the feature of SV structure (Fig. 3-1). Injecting an FGFR inhibitor to the SV region and observing the Akt activity and valve formation probably elucidate whether this hypothesis is correct or not. In addition, I cannot forget about the seminiferous epithelial cycle-dependent activation of Akt signaling in wild-type mouse ST region. Clarifying the factors of activating Akt signal in STs and the function of cyclical activation of Akt signaling is required. This cyclical activation of Akt signaling in ST region might have a relation with constitutive activation of Akt signaling in SV region. In the present study, I show that *Cyp26a1* is highly expressed in SV region and that data suggest that RA degradation is constitutively occurring in SV region, and balance between self-renewal and differentiation of SSCs is inclined more to self-renewal in SV region. I can

hypothesize that this situation is produced by constitutive activation of Akt signaling. If I apply this hypothesis to ST region, cyclical activation of Akt signaling in ST region may produce the cyclical balance of self-renewing and differentiation of SSCs.

RA signaling plays crucial roles during vertebrate development (Duester 2008, Niederreither & Dolle 2008). There is a concept of RA-FGF antagonism (Mercader *et al.* 2000) and it is applied to several mechanisms of development. In the chick embryo, FGF signaling has been shown to antagonize the RA gradient and to maintain the undifferentiated state of cells in the posterior part of the embryo throughout somitogenesis (Mathis *et al.* 2001, Diez del Corral & Storey 2004, Vermot & Pourquie 2005). On the research of mouse somitogenesis, conditional deletion in the mesoderm of *Fgfr1*, the only FGF receptor expressed in the mouse paraxial mesoderm, resulted in the absence of the RA-degrading enzyme CYP26 in the posterior part of the embryo (Wahl *et al.* 2007). In addition, on the research of mouse limb bud development, culturing of wild-type mouse forelimb bud in the presence of an inhibitor of the FGFR tyrosin kinase (SU5402) resulted in almost complete loss of *Cyp26b1* expression, and conversely, the implantation of FGF4-loaded beads into *Shh*-deficient mouse limb buds, of which the expression of *Cyp26b1* was reduced in the distal mesenchyme and RA activity was increased, resulted in striking restoration of *Cyp26b1* expression (Probst *et al.* 2011). Applying these findings to the present study, I can consider that FGF9 signaling in SV epithelia might activate the expression of *Cyp26a1* in SV epithelia. I will propose the hypothesis that there is RA-FGF antagonism in SCs, and in ST region, this antagonism is cyclically regulated by RA and FGF periodical secretion, while in SV

region, FGF signaling is more predominant by highly expression of *Fgfs* and degradation of RA.

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Fig. 3-1. Schematic representation of this study

In this study, I show the constitutive activation of Akt signaling in SV region, and this activation is considered to occur in a non-cell autonomous manner. *Cyp26a1* was highly expressed in SCs of SV. These data suggest that RA degradation is constitutively occurring in SV region, and balance between self-renewal and differentiation of SSCs is inclined more to self-renewal in SV region. *Fgf9* was highly expressed in RT region. Exogenous FGF9 could activate the Akt signaling in SCs. FGF9 from RT region might activate the Akt signaling in SCs of SV, and then SCs non-cell autonomously show the feature of SV epithelia.

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Kasane Imura-Kishi, D.V.M.

References

Abe T, Kiyonari H, Shioi G, Inoue K, Nakao K, Aizawa S & Fujimori T 2011 Establishment of conditional reporter mouse lines at ROSA26 locus for live cell imaging. *Genesis (New York, N.Y.: 2000)* **49** 579-590.

Aiyama Y, Tsunekawa N, Kishi K, Kawasumi M, Suzuki H, Kanai-Azuma M, Kurohmaru M & Kanai Y 2015 A Niche for GFRalpha1-Positive Spermatogonia in the Terminal Segments of the Seminiferous Tubules in Hamster Testes. *Stem cells (Dayton, Ohio)* **33** 2811-2824.

Altomare DA, Lyons GE, Mitsuuchi Y, Cheng JQ & Testa JR 1998 Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. *Oncogene* **16** 2407-2411.

Bowles J, Feng CW, Miles K, Ineson J, Spiller C & Koopman P 2016 ALDH1A1 provides a source of meiosis-inducing retinoic acid in mouse fetal ovaries. *Nature communications* **7** 10845.

Bowles J, Feng CW, Spiller C, Davidson TL, Jackson A & Koopman P 2010 FGF9 suppresses meiosis and promotes male germ cell fate in mice. *Developmental cell* **19** 440-449.

Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H & Koopman P 2006 Retinoid signaling determines germ cell fate in mice. *Science (New York, N.Y.)* **312** 596-600.

Brazil DP, Yang ZZ & Hemmings BA 2004 Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends in biochemical sciences* **29** 233-242.

Chen LY, Willis WD & Eddy EM 2016 Targeting the Gdnf Gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. *Proceedings of the National Academy of Sciences of the United States of America* **113** 1829-1834.

Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T & Hay N 2001 Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes & development* **15** 2203-2208.

Cho H, Thorvaldsen JL, Chu Q, Feng F & Birnbaum MJ 2001 Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *The Journal of biological chemistry* **276** 38349-38352.

Christakos S 2017 In search of regulatory circuits that control the biological activity of vitamin D. *The Journal of biological chemistry* **292** 17559-17560.

Chung CL, Lu CW, Cheng YS, Lin CY, Sun HS & Lin YM 2013 Association of aberrant expression of sex-determining gene fibroblast growth factor 9 with Sertoli cell-only syndrome. *Fertility and sterility* **100** 1547-54.e1-4.

Colvin JS, Green RP, Schmahl J, Capel B & Ornitz DM 2001 Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell* **104** 875-889.

Datta SR, Brunet A & Greenberg ME 1999 Cellular survival: a play in three Akts. *Genes & development* **13** 2905-2927.

de Rooij DG 2017 The nature and dynamics of spermatogonial stem cells. *Development (Cambridge, England)* **144** 3022-3030.

del Moral PM, De Langhe SP, Sala FG, Veltmaat JM, Tefft D, Wang K,

Warburton D & Bellusci S 2006 Differential role of FGF9 on epithelium and mesenchyme in mouse embryonic lung. *Developmental biology* **293** 77-89.

Diez del Corral R &Storey KG 2004 Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. *BioEssays : news and reviews in molecular, cellular and developmental biology* **26** 857-869.

Duester G 2008 Retinoic acid synthesis and signaling during early organogenesis. *Cell* **134** 921-931.

Dummler B, Tschopp O, Hynx D, Yang ZZ, Dirnhofer S & Hemmings BA 2006 Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. *Molecular and cellular biology* **26** 8042-8051.

Dym M 1974 The fine structure of monkey Sertoli cells in the transitional zone at the junction of the seminiferous tubules with the tubuli recti. *The American Journal of Anatomy* **140** 1-25.

Endo T, Freinkman E, de Rooij DG & Page DC 2017 Periodic production of retinoic acid by meiotic and somatic cells coordinates four transitions in mouse spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **114** E10132-E10141.

Endo T, Romer KA, Anderson EL, Baltus AE, de Rooij DG & Page DC 2015 Periodic retinoic acid-STRA8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. *Proceedings of the National Academy of Sciences of the*

United States of America **112** E2347-56.

Ezeasor DN 1986 Ultrastructural observations on the terminal segment epithelium of the seminiferous tubule of West African dwarf goats. *Journal of anatomy* **144** 167-179.

Fawcett DW &Dym M 1974 A glycogen-rich segment of the tubuli recti and proximal portion of the rete testis in the guinea-pig. *Journal of reproduction and fertility* **38** 401-409.

Feng CW, Bowles J & Koopman P 2014 Control of mammalian germ cell entry into meiosis. *Molecular and cellular endocrinology* **382** 488-497.

Figueiredo AF, Franca LR, Hess RA & Costa GM 2016 Sertoli cells are capable of proliferation into adulthood in the transition region between the seminiferous tubules and the rete testis in Wistar rats. *Cell cycle (Georgetown, Tex.)* **15** 2486-2496.

Franca LR, Hess RA, Dufour JM, Hofmann MC & Griswold MD 2016 The Sertoli cell: one hundred fifty years of beauty and plasticity. *Andrology* **4** 189-212.

Grasso M, Fuso A, Dovere L, de Rooij DG, Stefanini M, Boitani C & Vicini E 2012 Distribution of GFRA1-expressing spermatogonia in adult mouse testis. *Reproduction (Cambridge, England)* **143** 325-332.

Griswold MD 2016 Spermatogenesis: The Commitment to Meiosis. *Physiological Reviews* **96** 1-17.

Hara K, Nakagawa T, Enomoto H, Suzuki M, Yamamoto M, Simons BD & Yoshida S 2014 Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. *Cell stem cell* **14** 658-672.

Hasegawa K, Namekawa SH & Saga Y 2013 MEK/ERK signaling directly and

indirectly contributes to the cyclical self-renewal of spermatogonial stem cells. *Stem cells (Dayton, Ohio)* **31** 2517-2527.

Hasegawa K &Saga Y 2014 FGF8-FGFR1 signaling acts as a niche factor for maintaining undifferentiated spermatogonia in the mouse. *Biology of reproduction* **91** 145.

Hermo L &Dworkin J 1988 Transitional cells at the junction of seminiferous tubules with the rete testis of the rat: their fine structure, endocytic activity, and basement membrane. *The American Journal of Anatomy* **181** 111-131.

Hiramatsu R, Harikae K, Tsunekawa N, Kurohmaru M, Matsuo I & Kanai Y 2010 FGF signaling directs a center-to-pole expansion of tubulogenesis in mouse testis differentiation. *Development (Cambridge, England)* **137** 303-312.

Hogarth CA, Arnold S, Kent T, Mitchell D, Isoherranen N & Griswold MD 2015a Processive pulses of retinoic acid propel asynchronous and continuous murine sperm production. *Biology of reproduction* **92** 37.

Hogarth CA, Evans E, Onken J, Kent T, Mitchell D, Petkovich M & Griswold MD 2015b CYP26 Enzymes Are Necessary Within the Postnatal Seminiferous Epithelium for Normal Murine Spermatogenesis. *Biology of reproduction* **93** 19.

Hogarth CA &Griswold MD 2010 The key role of vitamin A in spermatogenesis. *The Journal of clinical investigation* **120** 956-962.

Ishii K, Kanatsu-Shinohara M, Toyokuni S & Shinohara T 2012 FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. *Development (Cambridge, England)* **139** 1734-1743.

Jacobsen B &Ploug M 2008 The urokinase receptor and its structural homologue C4.4A in human cancer: expression, prognosis and pharmacological inhibition. *Current medicinal chemistry* **15** 2559-2573.

Johnson A, Gomes W & Vandemark N 1970 The testis. Volume I. Development, anatomy and physiology. *The testis.Volume I.Development, anatomy and physiology.*

Johnston DS, Olivas E, DiCandeloro P & Wright WW 2011 Stage-specific changes in GDNF expression by rat Sertoli cells: a possible regulator of the replication and differentiation of stem spermatogonia. *Biology of reproduction* **85** 763-769.

Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S & Shinohara T 2003 Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biology of reproduction* **69** 612-616.

Kandel ES &Hay N 1999 The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Experimental cell research* **253** 210-229.

Kishi K, Uchida A, Takase HM, Suzuki H, Kurohmaru M, Tsunekawa N, Kanai-Azuma M, Wood SA & Kanai Y 2017 Spermatogonial deubiquitinase USP9X is essential for proper spermatogenesis in mice. *Reproduction (Cambridge, England)* **154** 135-143.

Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD & Page DC 2006 Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proceedings of the National Academy of Sciences of the United States of America* **103** 2474-2479.

Kriegbaum MC, Jacobsen B, Fuchtbauer A, Hansen GH, Christensen IJ, Rundsten CF, Persson M, Engelholm LH, Madsen AN, Di Meo I, Lund IK, Holst B,

Kjaer A, Laerum OD, Fuchtbauer EM & Ploug M 2016 C4.4A gene ablation is compatible with normal epidermal development and causes modest overt phenotypes. *Scientific reports* **6** 25833.

Kubota H, Avarbock MR & Brinster RL 2004 Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **101** 16489-16494.

Kulibin AY &Malolina EA 2016 Only a small population of adult Sertoli cells actively proliferates in culture. *Reproduction (Cambridge, England)* **152** 271-281.

Li H &Clagett-Dame M 2009 Vitamin A deficiency blocks the initiation of meiosis of germ cells in the developing rat ovary in vivo. *Biology of reproduction* **81** 996-1001.

Li H, MacLean G, Cameron D, Clagett-Dame M & Petkovich M 2009 Cyp26b1 expression in murine Sertoli cells is required to maintain male germ cells in an undifferentiated state during embryogenesis. *PloS one* **4** e7501.

Li S, Lan ZJ, Li X, Lin J & Lei Z 2014 Role of postnatal expression of fgfr1 and fgfr2 in testicular germ cells on spermatogenesis and fertility in mice. *Journal of reproduction & infertility* **15** 122-133.

Lindner SG 1982 On the morphology of the transitional zone of the seminiferous tubule and the rete testis in man. *Andrologia* **14** 352-362.

Lord T &Oatley JM 2017 A revised Asingle model to explain stem cell dynamics in the mouse male germline. *Reproduction (Cambridge, England)* **154** R55-R64.

MacLean G, Li H, Metzger D, Chambon P & Petkovich M 2007 Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. *Endocrinology* **148**

4560-4567.

Mathis L, Kulesa PM & Fraser SE 2001 FGF receptor signalling is required to maintain neural progenitors during Hensen's node progression. *Nature cell biology* **3** 559-566.

Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M & Sariola H 2000 Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science (New York, N.Y.)* **287** 1489-1493.

Mercader N, Leonardo E, Piedra ME, Martinez-A C, Ros MA & Torres M 2000 Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. *Development (Cambridge, England)* **127** 3961-3970.

Miller DL, Ortega S, Bashayan O, Basch R & Basilico C 2000 Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. *Molecular and cellular biology* **20** 2260-2268.

Min H, Danilenko DM, Scully SA, Bolon B, Ring BD, Tarpley JE, DeRose M & Simonet WS 1998 Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. *Genes & development* **12** 3156-3161.

Niederreither K &Dolle P 2008 Retinoic acid in development: towards an integrated view. *Nature reviews.Genetics* **9** 541-553.

Nykanen M 1979 Fine structure of the transitional zone of the rat seminiferous tubule. *Cell and tissue research* **198** 441-454.

Oakberg EF 1956a A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *The American Journal of Anatomy* **99** 391-413.

Oakberg EF 1956b Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *The American Journal of Anatomy* **99** 507-516.

Ohuchi H, Hori Y, Yamasaki M, Harada H, Sekine K, Kato S & Itoh N 2000 FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. *Biochemical and biophysical research communications* **277** 643-649.

Ornitz DM &Itoh N 2015 The Fibroblast Growth Factor signaling pathway. *Wiley interdisciplinary reviews.Developmental biology* **4** 215-266.

Osman DI 1978 On the ultrastructure of modified Sertoli cells in the terminal segment of seminiferous tubules in the boar. *Journal of anatomy* **127** 603-613.

Osman DI 1979 A comparative ultrastructural study on typical and modified Sertoli cells before and after ligation of the efferent ductules in the rabbit. *Anatomia, Histologia, Embryologia* **8** 114-123.

Potter SJ &DeFalco T 2017 Role of the testis interstitial compartment in spermatogonial stem cell function. *Reproduction (Cambridge, England)* **153** R151-R162.

Probst S, Kraemer C, Demougin P, Sheth R, Martin GR, Shiratori H, Hamada H, Iber D, Zeller R & Zuniga A 2011 SHH propagates distal limb bud development by enhancing CYP26B1-mediated retinoic acid clearance via AER-FGF signalling.

Development (Cambridge, England) **138** 1913-1923.

Roosen-Runge EC 1961 The rete testis in the albino rat: its structure, development and morphological significance. *Acta Anatomica* **45** 1-30.

Russell L, Ettlin R, Sinha Hikim A & Clegg E 1990 Histological and histopathological evaluation of the testis. *Cache River Press, Clearwater*.

Russell LD &Griswold MD 1993 The Sertoli cell / edited by Lonnie D. Russell, Michael D. Griswold. xxv, 801 p.

Sato T, Aiyama Y, Ishii-Inagaki M, Hara K, Tsunekawa N, Harikae K, Uemura-Kamata M, Shinomura M, Zhu XB, Maeda S, Kuwahara-Otani S, Kudo A, Kawakami H, Kanai-Azuma M, Fujiwara M, Miyamae Y, Yoshida S, Seki M, Kurohmaru M & Kanai Y 2011 Cyclical and patch-like GDNF distribution along the basal surface of Sertoli cells in mouse and hamster testes. *PloS one* **6** e28367.

Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N & Kato S 1999 Fgf10 is essential for limb and lung formation. *Nature genetics* **21** 138-141.

Sharpe RM, McKinnell C, Kivlin C & Fisher JS 2003 Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction (Cambridge, England)* **125** 769-784.

Shinohara T, Orwig KE, Avarbock MR & Brinster RL 2003 Restoration of spermatogenesis in infertile mice by Sertoli cell transplantation. *Biology of reproduction* **68** 1064-1071.

Shinomura M, Kishi K, Tomita A, Kawasumi M, Kanezashi H, Kuroda Y,

Tsunekawa N, Ozawa A, Aiyama Y, Yoneda A, Suzuki H, Saito M, Picard JY, Kohno K, Kurohmaru M, Kanai-Azuma M & Kanai Y 2014 A novel Amh-Treck transgenic mouse line allows toxin-dependent loss of supporting cells in gonads. *Reproduction (Cambridge, England)* **148** H1-9.

Small CL, Shima JE, Uzumcu M, Skinner MK & Griswold MD 2005 Profiling gene expression during the differentiation and development of the murine embryonic gonad. *Biology of reproduction* **72** 492-501.

Sugimoto R, Nabeshima Y & Yoshida S 2012 Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. *Mechanisms of development* **128** 610-624.

Sun W, Chen L, Zhang W, Wang R, Goltzman D & Miao D 2015 Active vitamin D deficiency mediated by extracellular calcium and phosphorus results in male infertility in young mice. *American journal of physiology.Endocrinology and metabolism* **308** E51-62.

Takase HM &Nusse R 2016 Paracrine Wnt/beta-catenin signaling mediates proliferation of undifferentiated spermatogonia in the adult mouse testis. *Proceedings of the National Academy of Sciences of the United States of America* **113** E1489-97.

Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K, Ogonuki N, Jijiwa M, Takahashi M, Ogura A & Shinohara T 2015 Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. *Stem cell reports* **4** 489-502.

Thatcher JE &Isoherranen N 2009 The role of CYP26 enzymes in retinoic acid

clearance. *Expert opinion on drug metabolism & toxicology* **5** 875-886.

Uchida A, Kishi K, Aiyama Y, Miura K, Takase HM, Suzuki H, Kanai-Azuma M, Iwamori T, Kurohmaru M, Tsunekawa N & Kanai Y 2016 In vivo dynamics of GFRalpha1-positive spermatogonia stimulated by GDNF signals using a bead transplantation assay. *Biochemical and biophysical research communications* **476** 546-552.

van Pelt AM &de Rooij DG 1990 Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. *Biology of reproduction* **43** 363-367.

Vermot J &Pourquie O 2005 Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos. *Nature* **435** 215-220.

Vernet N, Dennefeld C, Rochette-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB & Mark M 2006 Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology* **147** 96-110.

Wahl MB, Deng C, Lewandoski M & Pourquie O 2007 FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. *Development (Cambridge, England)* **134** 4033-4041.

Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ & Luo Y 2012 RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *The Journal of molecular diagnostics : JMD* **14** 22-29.

Yang ZZ, Tschopp O, Hemmings-Mieszczak M, Feng J, Brodbeck D, Perentes E & Hemmings BA 2003 Protein kinase B alpha/Akt1 regulates placental development and

fetal growth. *The Journal of biological chemistry* **278** 32124-32131.

Yao HH, Ungewitter E, Franco H & Capel B 2015 2 - Establishment of fetal Sertoli cells and their role in testis morphogenesis. In *Sertoli Cell Biology (Second Edition),* pp 57-79. Ed. MD Griswold, Oxford: Academic Press.

Yoshida S, Sukeno M & Nabeshima Y 2007 A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science (New York, N.Y.)* **317** 1722-1726.