Analyses of gene regulatory networks involved in murine oocyte formation

Fukuda, Kurumi

Doctor of Philosophy

Department of Genetics

School of Life Science

SOKENDAI

(The Graduate University for Advanced Studies)

Introduction

Mammalian oogenesis consists of two genetically distinct events. One is meiosis, which starts in germline cysts in embryonic ovaries, and the other is folliculogenesis occurred after birth. The latter process is initiated by the event termed primordial follicle formation in neonatal ovaries, by which germline cysts are separated and oocytes are enclosed by their supporting somatic cells, pre-granulosa cells. The interaction between germ cells and granulosa cells is known to be important to generate functional oocytes. However, the underlying mechanisms remain elusive.

In my thesis study, I addressed the mechanisms of oogenesis after birth via two different perspectives regarding germ cells and somatic cells. In Chapter I, I described my investigation of the function of a germ cell-specific factor, DAZL, in the postnatal ovary, and in Chapter II, I discussed my analysis of gene expression changes in somatic cells focusing on the pre-granulosa cell lineage during primordial follicle formation by using developed cell collecting method.

Result

In Chapter I, I focused on an RNA-binding protein implicated in the translational promoting factor, DAZL, which is known to be a critical factor for meiotic

progression and is expected to play a role in folliculogenesis. First, I examined *Dazl* function in the postnatal ovary by generating postnatal oocyte-specific *Dazl* knockout mice. However, I unexpectedly found that the *Dazl* gene is dispensable for oocyte differentiation in postnatal ovaries as mutants were fertile and produced a normal number of pups. In addition, DAZL protein expression was not detected in the postnatal ovary, whereas *Dazl* mRNA was continuously observed, suggesting that DAZL is post-transcriptionally suppressed after birth.

Next, I asked whether DAZL translation was suppressed via its 3'-UTR sequence. To address this, I used BAC transgenic mice, in which 3'UTR element was franked with Frt sequence and it can be removed by crossing with Flp mice. Thus, utilizing this mouse, I removed the *Dazl* 3'-UTR and examined DAZL expression. As a result, DAZL expression was increased in the 3'-UTR-deleted transgenic ovaries, even postnatally, suggesting that DAZL translation is suppressed in a 3'-UTR-dependent manner. Furthermore, the increased DAZL expression caused abnormalities in the oocytes because the mothers bearing *Dazl* gene without 3'-UTR delivered a reduced number of pups. I found that the defect was due to the failure of pre-implantation development, indicating that the 3'-UTR-dependent suppression of DAZL in postnatal oocytes is required for making functional oocytes. Based on these results, I concluded

that the proper switching of DAZL expression from “on” in the embryonic stage to “off" in the postnatal stage is essential to produce functional oocytes.

In Chapter-II, to extract genes involved in the primordial follicle formation, I performed transcriptome analysis of pre-granulosa cell progenitor cells. For this purpose, I used *Lgr5*-EGFP mice because *Lgr5* is known to mark pre-granulosa cell progenitor cells. However, it has problem because *Lgr5* also marks ovarian epithelial cells. To collect only progenitor cells, I developed a method the gonads treated with Mitotracker briefly to stain only ovarian epithelial cells. It enables to separate ovarian epithelial cell and progenitor cells. I collected *Lgr5*-positive pre-granulosa cell progenitors from wild-type and germ cell-deficient ovaries using a FACS sorting method and performed transcriptome analyses. Through these comparative analyses, I identified increased and decreased genes associated with pre-granulosa cell differentiation. The most marked changes I found during primordial follicle (PF) formation were the significant downregulation of Wnt and TGF-beta signaling pathway genes, and the upregulation of Notch and PI3K pathway genes. Although *Lgr5*-positive pre-granulosa progenitors developed in the absence of germ cells, oocytes influenced pre-granulosa cell differentiation after birth, suggesting that crosstalk between germ cells and *Lgr5*-positive cells is important for the differentiation of pre-granulosa cells. Thus, I

further classified the genes with increased or decreased expression during PF formation as germ cell-dependent or -independent ones by comparing transcriptome results of germ cell defective mutants, *Figla* and *Nanos3* knockout. Based on this classification, gene upregulation was largely dependent on germ cells, whereas the downregulated genes, which comprised putative factors involved in the retention of germline cysts, were regulated in both germ cell-dependent and -independent manners. These results provide insight into the gene regulatory networks functioning in differentiating pre-granulosa cells during primordial follicle formation. Moreover, they will help further analyses to identify unknown factors required for pre-granulosa cell differentiation.

Discussion

My thesis study revealed dynamic changes from the embryonic to postnatal stages during oocyte development such as DAZL expression regulation in oocytes and gene expression changes during pre-granulosa cell differentiation in somatic cells. By focusing on different events related to oocyte development, as described in Chapters I and II, my study helps to clarify the complicated regulatory mechanisms at the transcriptional and post-transcriptional levels necessary for making a functional oocyte.

The cause of pre-implantation defects in *Dazl 3F;Flp*

My results in the chapter I, suggest that the suppression of *Dazl* translation in follicular oocytes is required for producing the proper number of progenies. However, why excess DAZL expression causes defective pre-implantation development remains unclear. As DAZL has been implicated in the positive regulation of translation, it is possible that the observed defect may be due to abnormal translational promotion. However, my result about expression one of known DAZL target, *Tex19.1* is downregulated in the mRNA level but not in protein. Alternatively, it is also possible that excess DAZL abnormally suppresses its target RNAs because DAZL works as a component of stress granules, cytoplasmic RNP granules involved in translational suppression or mRNA storage, in the testis. In this case, *Tex19.1* expression reduction is somehow reasonable but other known targets were not changed its mRNA expression. In any cases, further comprehensive analysis such as transcriptome and proteome analysis are required for downstream event which caused pre-implantation defects in *Dazl3F; Flp.*

Candidates of important pathways for proper PF formation

In the chapter II, the most significant changes I noticed during PF formation was down-regulation of Wnt and TGF-beta signaling related genes, and up-regulation of Notch and PI3K signaling pathway genes and TFs known to be involved in

folliculogenesis *(Dmrta1*, *Smad3*). In addition, previous studies suggested that Wnt signaling inhibits FSH target genes and steroid production associated with maturation and differentiation of rat ovarian follicles, while FSH is also implicated in promoting cyst breakdown and PF formation by mouse organ culture study, implying that Wnt signaling related genes in my data may have a role in retaining pre-G cells by suppressing FSH related pathways. Therefore, I expect that Wnt signaling related factors are major regulators required for protecting germ line cyst from the cyst breakdown. Though Wnt signaling factors were classified as germ cell-dependent, I think that germ line cyst may be retained via both internal and external signals because *Lgr5*-positive cells were emerged in germ cell less mutants. Other than known PF formation promoting genes, the up-regulated genes include important genes for follicle development such as *Smad3* and *Igf1r*. IGF1R recruits the Ras/MAPK and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways and also is known to have the cross-talk with the EGFR pathway. As factors included in PI3K and EGFR pathway were contained in my list, these signaling pathways may have roles in follicular development in the downstream of IGF1R though it is possible these factor have a function also in PF formation promotion. Besides, the number of germ cell-dependent genes was two-fold higher in up-regulated gene during PF formation

than that of germ cell-independent ones, supports the idea that differentiation of pre-G is strongly affected by the interaction with germ cells.

Analysis of PF maintenance related genes

I found that *Lgr5*-positive pre-G progenitors in cortical region emerged even in the absence of the germ cells. Therefore, I speculate that *Lgr5*-positive cells can be separated into two cell groups based on their developmental fates; one can differentiate to FOXL2-positive cells in the embryonic stage, which is germ cell-independent (not focused on this study), while others maintain *Lgr-5* expression until PF formation and finally differentiate to FOXL2-positive cell after birth, which process must be germ cell-dependent. My transcriptome analyses using mutant ovaries identified possible candidate genes involved in each process. I indicated that Wnt signaling might be involved in the maintenance of pre-G progenitor in a germ cell-dependent pathway but it is still largely unknown how Wnt signaling works. Whereas it is known that WNT4 have a role in maintaining E-cadherin junctions between oocytes inside cyst. In addition, although I mainly focused on *Lgr5*-positive cells, LGR5 function during PF formation is still unknown because *Lgr5*-KO causes neonatal lethality. For further analysis, I like to identify pre-G progenitor specific genes to use as markers and to create specific Cre-expressing mouse lines. As candidates, I extracted 55 genes including *Lgr5* among

171 down regulated genes. I expect that those contain genes not only useful for as the pre-G marker but also have important roles in the maintenance of pre-G progenitor.