論文の内容の要旨

応用動物科学専攻

平成 24 年博士課程進学

藤岡良江

指導教員　内藤邦彦

論文題　Analysis on the functions of ubiquitin-proteasome-pathway factors in porcine oocyte maturation process

(ユビキチン/プロテアソーム経路関連因子のブタ卵成熟過程における機能の解析)

The oscillation of protein level such as of cyclin B (CCNB), a regulatory subunit of M-phase promotind factor (MPF), is essential to mitotic and meiotic cell cycle progression, and thus, the analysis of protein degradation mechanism is critical for understanding the integrative regulation of oocyte meiosis (also called as oocyte maturation). Ubiquitin-proteasome-pathway (UPP) is one of the protein-degradation-pathways, and within this pathway, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) are working together to ubiquitinate the substrate which is then degraded by proteasome. CCNB ubiquitination is known to be performed by APC/C as E3 and UBE2C and UBE2S as E2s. Addition to these, the activity of APC/C is known to be inhibited by the factors called EMI1 and EMI2. However, most of the knowledges about UPP are of mitotic cell cycle and the reports focusing on meiotic cell cycle are restricted to *Xenopus* and mouse oocytes, making it difficult to create the thorough understanding of UPP importance to meiotic cell cycle. Adding to this, those previous studies had focused on only one section of whole oocyte maturation process such as only the germinal vesicle breakdown (GVBD), which marks the initiation of meiotic resumption in oocyte, or meiotic arrest at the second meiotic metaphase (MII). Thus, comprehensive studies using mammalian oocytes other than mouse are required to unravel the functions and roles of EMI and UBE2 in meiotic regulation of oocytes. In this study, I focused on UPP factors, EMI1, EMI2, UBE2C, and UBE2S,

and investigated their functions and roles in porcine oocyte maturation. I had used RNA injection method to overexpress and downregulate the factors by injecting mRNA and antisense RNA (asRNA), respectively.

Chapter 1: Effects of EMI regulation to porcine oocyte maturation

From the EMI overexpression, the timing of GVBD was accelerated with the CCNB accumulation during this period, supporting the reported observation that EMI1 and EMI2 had inhibited CCNB degradation. Previous studies on mouse had shown that EMI overexpression would initiate early GVBD, and together with the present result, it indicates that mammalian EMIs have the ability to initiate early GVBD. As for the EMI downregulation, the GVBD was delayed in EMI1 downregulation while no change in the timing of GVBD was observed for EMI2 downregulation, indicating that EMI2 may not be present in oocyte during this period. EMI1 overexpressed oocytes were observed to reach to MII whereas majority of EMI2 overexpressed oocytes had been arrested at first meiotic metaphase (MI). This result supported that EMI2 does not exist before MI since if it does exist, then the CCNB degradation is inhibited, making oocytes unable to go through MI to MII transition.

Although normally cultured oocytes are arrested at MII until fertilization, the inhibition of EMI2 provoked release from this arrested state and formed pronucleus (PN) while EMI1 downregulated oocytes were held at MII. Concerning this matter, the parthenogenetic activation was done to assess the effect of EMI overexpression to PN formation. The result showed that PN formation was inhibited only in EMI2 overexpression and EMI1 overexpression did not had any effect on PN formation. The expression analysis revealed that only EMI2 was able to firmly exist while EMI1 was being degraded in MII phase oocytes. These results confirmed that only EMI2 is functioning in MII phase oocytes. Considering the findings made in chapter 1, it can be concluded that EMI1 regulate the initiation of GVBD by provoking the accumulation of CCNB while EMI2 is essential to MII maintenance and degraded after oocyte activation to form PN.

Chapter 2: Effects of UBE2 regulation on porcine oocyte maturation

When the effect of UBE2 downregulation was assessed toward GVBD initiation, UBE2C downregulation had initiated early GVBD while UBE2S downregulation did not effected GVBD. UBE2C and UBE2S contribute to ubiquitination differently with UBE2C contributing to mono-ubiquitination and UBE2S to poly-ubiquitination. Therefore, the downregulation of UBE2C inhibits both mono- and poly-ubiquitination while UBE2S downregulation

inhibits only poly-ubiquitination. Thus, the results of UBE2 downregulation had shown that the poly-ubiquitination was not necessary for the CCNB degradation during GVBD period. Delayed GVBD by the UBE2C overexpression was consistent with this speculation. In *Xenopus* oocyte, CCNB is reported to be degraded only by mono-ubiquitination, and thus, the present results indicated that same can be applied to porcine oocytes. Unexpectedly for UBE2S overexpression, it had initiated early GVBD with the CCNB degradation. This is further discussed in chapter 3.

For the effect on MII phase, the formation of PN was observed only in UBE2S overexpressed oocytes while UBE2C overexpressed oocytes remained at MII. This implied that the contribution of UBE2S and poly-ubiquitination is required for the CCNB degradation during MII phase. The PN formation was inhibited in both UBE2 downregulated oocytes when parthenogenetically activated, confirming the idea that the poly-ubiquitination being essential to CCNB degradation in MII phase oocytes. Considering the finding made in chapter 2, it was suggested that the configuration of ubiquitination required toward the substrate degradation differ among stages in oocyte maturation.

Chapter 3: Studies on the mechanism involves with early GVBD initiated by Flag-UBE2S overexpression

From chapter 2, the result revealed the UBE2S overexpression initiated early GVBD despite of inhibited accumulation of CCNB. Concerning this, I investigated whether the early GVBD was triggered by UBE2S's function as E2 or not. For this purpose, APC/C-binding-region-omitted UBE2S, UBE2S⊿APC, was expressed in oocytes, since UBE2S must bind to APC/C in order to function as E2 . The result revealed that overexpression of UBE2S⊿APC did not effected GVBD, indicating the requirement of UBE2S binding to APC/C for early GVBD triggered by UBE2S overexpression. This further implied some substrate is being ubiquitinated by UBE2S-APC/C interaction, and delineated the existence of a possible substrate which the degradation could initiate GVBD.

As being one of the candidate substrate, WEE1B degradation was observed. WEE1B is a kinase inactivating MPF, which activity can trigger the initiation of GVBD, and thus WEE1B degradation should induce the early GVBD. However, the result showed UBE2S does not trigger WEE1B degradation. Next candidate was AKAP5. AKAP5 is an anchor protein holding protein kinase A (PKA) to oocyte cytoplasm. In previous study, the downregulation of AKAP5 in porcine oocytes triggered GVBD by the translocation of PKA regulatory subunit, PKA-RII into GV. Thus, if AKAP5 were to be degraded by UBE2S overexpression, the translocation of PKA-RII could be observed. The results revealed that no PKA-RII translocation into GV was observed when UBE2S was co-overexpressed, indicating that UBE2S does not contribute to AKAP5 degradation. Considering the finding made in chapter 3, it was confirmed that the early GVBD

initiated by UBE2S overexpression was possibly induced by ubiquitinating some substrate. However, the degrade substrate was neither WEE1B nor AKAP5, indicating the presence of some other candidate substrate for triggering the early GVBD. Addition to this, there still is another possibility that the ubiquitination via UBE2S-APC/C interaction is not contributing to protein degradation but to some other pathway, such as translation modification and signal transduction. Thus, there still needs the investigations to unravel the unknown mechanism underlying early GVBD initiated by UBE2S overexpression.

From this study, I was able to concisely examine the functions and roles of UPP factors throughout oocyte maturation. Furthermore, the study was able to associate the contribution of UBE2C and UBE2S as the necessity of mono- and poly- ubiquitination during the certain stages of oocyte maturation. Addition to this, the present study also was able to reveal the unexpected early GVBD, which was never annotated in either *Xenopus* or mouse oocyte. Thus, this study indicated some characteristics of UPP factors which were reported in mouse was also conserved in porcine oocytes and could be dilated in mammals, besides the newly discovered phenomenon that may contribute to the further understanding of oocyte maturation.