A Thesis for the Degree of Master of Science

Molecular and Signal Signatures in the Dormant Avian Embryo

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SUMMARY

In most of the avian species, the early embryo suspends development after oviposition because of the low ambient temperature, which does not meet the thermal requirements for development. A periodic developmental layoff in the embryo is known as “cold torpor”, and it enables to reduce energy expenditure during periods of stressful events such as cold exposure. Immediately after oviposition, the early avian embryo undergoes cold torpor before the major development occurs, but it is able to endure some period of cold exposure. At the cellular level, it is known that shortly after exposure to thermal stress, various protective or destructive molecular signals are activated. Some examples of the representative molecular changes that occur under stressful conditions are unfolded protein response (UPR) triggered by endoplasmic reticulum (ER) stress and stress-activated protein kinase (SAPK) signaling. Multiple cellular process, such as apoptosis or cell cycle arrest occur in response to the activation of the ER stress or SAPK signaling initiation, thus promoting the cell death. In spite of the understanding of stress activated molecular mechanisms in various types of cells, the exact molecular signatures occurring in the avian embryo during cold torpor are not clearly elucidated to date. Here we investigated the molecular signaling signatures that occur in the dormant avian embryo, especially the mechanisms related to stress response and energy conservation.

To investigate the level of ER stress and SAPK signaling, and its effect on the cellular integrity in the post-ovipositional avian embryo,

molecular analysis was conducted using the avian embryos that were ovipositioned and cooled at 16°C during several time periods. First of all the internal egg temperature was measured to observe the timing of cold stress initiation in the blastoderm. This indicated that hypothermia occur with in 4 hours post-oviposition, which is caused by at least 20°C decline in the egg internal temperature, and enough to initiate stress-activated molecular signals.

To conduct studies on the degree of ER stress and SAPK signaling during avian embryo dormancy, the mRNA expression pattern was screened in various time points after oviposition. As a result, the mRNA of ER stress, SAPK signaling and apoptosis related genes were gradually up-regulated during the post-ovipositional periods, especially the gene expression was significantly up-regulated after 7 days of storage, indicating the strong activation of ER stress and SAPK signaling related genes. Furthermore, to investigate the degree of protein expression change of ER stress and SAPK signaling related molecules, western blot analysis was conducted with proteins obtained from blastoderm that were storaged at 16°C for several time points post-oviposition. Western blot results indicated that SAPK signal phosphorylation, especially JNK and p38 signaling pathway, was stronger as the duration of cold torpor increases in the avian embryo, and is strongest at 7 days of storage. Similarly, the ER stress effector protein IRE-1 phosphorylation increased during the cold torpor period. Identical analysis was performed with duck blastodermal proteins, and it has shown identical results as chicken.

To investigate the cellular process that occur in response to the activation of ER stress and SAPK signaling, the degree of cell cycle progression was measured by gene expression profiling and propidium iodide (PI) staining. The results indicated that shortly after oviposition, genes related to the progression of cell cycle were significantly down regulated, especially at 1 day of storage. Additionally, the distribution of cells throughout the cell cycle phases were investigated by PI staining, accordingly the results indicated that shortly after oviposition, most of the blastodermal cells are in the G_0/G_1 phase. However, the cell cycle was arrested at the G_2/M phase at 7 days of storage, which shows that the blastodermal cells suspend the progression of cell cycle during cold torpor. Accordingly, the mRNA expression pattern of the purine/pyrimidine metabolism related genes were investigated to observe the extent of nucleotide metabolism in the dormant embryo, and the results indicated that the expression of nucleotide metabolism related genes are down-regulated at 7 days of storage.

Another cellular process that occurs in response to stress is apoptosis, which is a form of programmed cell death induced by various cellular signals as ER stress and SAPK signaling. AnnexinV-PI staining was conducted to investigate the progression of apoptotic cell death in the blastoderm and measure proportion of apoptotic cells. The results shown that early apoptotic cells rise after 7 day of storage at 16°C, while late apoptotic cells does not. Nonetheless, after incubation of the embryos at 37.5°C for 4 hours, the early and late apoptotic cells were cleared and amount of live cells increased. The TUNEL assay results show that there is little damage to the DNA during storage,

indicating that the early apoptotic cells in the blastoderm does not affect DNA integrity.

In conclusion, after oviposition, various stress-related molecular signals are active, which affects the molecular status of the blastoderm by arresting the cell cycle and inducing early apoptosis. However, the avian embryo is able to prohibit the progression of apoptosis to late phase, and minimize damage to genetic information. This result suggests that the avian embryo is able to actively protect itself from external stress during the dormant period.

Key words: Egg storage, dormancy, cold torpor, ER stress, SAPK signaling, apoptosis, cell cycle

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LIST OF ABBREVIATIONS

ER	Endoplasmic Reticulum
UPR	Unfolded protein response
SAPK	Stress-activated protein kinase
MAPK	Mitogen-activated protein kinase
JNK	c-Jun-N-terminal kinases
ERK	Extracellular signal-regulated kinases
HSP	Heat shock protein
HSF	Heat shock factor
PCD	Programmed cell death
FADD	Fas-associated death domain protein
TNF	Tumor necrosis factor
MPT	Mitochondrial permeability transition pore
CAD	Caspase-activated DNase
IRE1	Inositol-requiring enzyme 1
miRNA	Micro RNA
PERK	PRKR-like endoplasmic reticulum kinase
eIF2α	Eukaryotic initiation factor α
ASK1	Apoptosis signal-regulating kinase
CHOP	C/EBP homologous protein
SG	Stress granules
CDK	Cyclin-dependent kinases
ROS	Reactive oxygen species
CIRP	Cold-inducible RNA-binding protein
PRPP	Phosphoribosyl-1-pyrophosphate

HUVEC	Human umbilical vein endothelial cell
AMP	Adenosine monophosphate
GMP	Guanosine monophosphate
CAD	Carbamoyl-phosphate synthetase-aspartate carbamoyltransferase-dihydroorotase
PI	Propidium iodide

CHAPTER 1

Introduction

Exposure to acute cold stress is common in most avian species, because of its oviparous nature (Patterson et al., 2008). The ambient temperature of where the egg is laid will vary, but has at least 10°C difference from the oviductal temperature (41°C). The ability of an animal to endure temperature decline is called “cold torpor”, and this phenomenon occurs mostly in hibernating animals or reptilian and avian embryos (Ewert, 1991). The characteristic of cold torpor is to suspend the development during the stressed period, and defend the embryo from damage that could be caused by low temperature. In many avian species, brooding of eggs occur only after the last egg in the clutch has laid (Winkler and Walters, 1983). Therefore, the first egg laid in the clutch will experience a period of moderate to harsh cold torpor, compared to the last egg that is immediately incubated. For example, the chicken (*Gallus gallus*) eggs are industrially stored at 10°C to 16°C up to 14 days without significantly affecting the hatchability of the chicks (Fasenko, 2007). There were several studies that investigated the internal changes in the eggs during storage, such as pH elevation in the albumen or egg mass decline, but yet there is handful information on the molecular changes in the avian embryo during dormancy (Lapao et al., 1999; Rocha et al., 2013; Walsh et al., 1995). As demonstrated, the avian embryo has unique characteristics of dormancy during embryonic development, but the underlying molecular changes or mechanisms are yet to be investigated. Therefore, studying the molecular phenomenon occurring in the dormant avian embryo will be valuable to understand the unknown mechanisms that maintain the embryo’s homeostasis during dormancy.

In the cellular level, the most immediate change when external stress (temperature, oxygen availability) is present is the protein misfolding. To prevent the misfolding of protein under stressful events, the unfolded protein response (UPR) is initiated to maintain the correct folding of proteins, and this process manages the correct level of protein influx in the endoplasmic reticulum (ER) (Cao and Kaufman, 2012). Once there is overload of misfolded proteins in the ER, the ER stress mechanism is active, which is controlled by three effectors on the ER lumen characterized as PERK, IRE-1 and ATF6 (Sano and Reed, 2013). Each effector utilizes a unique method for the initiation of apoptosis as the final cellular process.

ER stress is also closely related to stress-activated protein kinase (SAPK) signaling, which is a conserved cellular signaling mechanism to conduct information about the internal and external cellular stress (Cowan, 2003). SAPK signaling can be activated by the ER stress effector IRE-1, which phosphorylates the MAP3K and initiates the signaling (Chen and Brandizzi, 2013). To combine together, the ER stress and SAPK signaling affects the cellular status during stressful events, and this both processes acts to initiate apoptosis, which is a form of programmed cell death. The initiation of apoptosis marks the start of demolition in the cell.

Apoptosis is a unique cellular process, because it is a tightly controlled mechanism of multiple cell death related genes such as caspases. Apoptosis is marked by the phenotypical and genetic change in the cell, and both change are important in the detection of apoptosis. Phenotypically an apoptotic cell is distinguished by the plasma membrane blebbing, chromatin condensation and margination

and formation of apoptotic bodies (Krysko et al., 2008). Once the apoptotic process is active, the cell is most likely to undergo programmed cell death. However, in some cases, an apoptotic cell is able to reverse itself from apoptosis, only when it is in the early apoptotic phase, where the cell membrane integrity is still preserved and the executive caspases are not activated (Tang et al., 2012).

In addition to the apoptotic phase activation, cell cycle is also affected by the stress signaling, especially by being arrested at a specific phase under stress. The cell cycle is one of the most important mechanisms in the biological processes, and is controlled by multiple pathways and proteins to prevent errors in the cycling. It is mainly related to the cell division, and DNA replication that includes the G_0, G_1, S, G_2 and M phase, and is progressed through several checkpoints that assure that the cell cycle is correctly performed. Under stressful conditions, it is known that the cell cycle is arrest at specific points, and especially during cold stress, it is known that the cell cycle is arrested at G_2/M in various types of cultured cells (Rieder and Cole, 2014). Moreover, the arrest at G_2/M phase induces the activation of apoptosis through the phosphorylation of MAP3K of the SAPK signaling, thus increasing the cell death signals (Wang et al., 2000).

In summary, shortly after oviposition the avian embryo is known to be exposed to cold stress, which can possibly activate various cellular and molecular stress signals. However, the exact molecular mechanisms and signaling signatures are not elucidated in the dormant avian embryo. Therefore, it will be valuable to investigate the molecular changes that occur in the dormant avian blastoderm, thus

providing information about the stress-resistant nature of the avian blastoderm.

CHAPTER 2

Literature review

1. Embryonic dormancy in animal embryos

Oviparous species, represented by animals such as reptiles, fish and birds, lay egg with little or no embryonic development within the mother. After oviposition, the embryonic development can be divided into active growth and differentiation phase and the period with relatively inactive growth with suspended development. In general, until the egg is incubated at optimal temperature, the embryonic development is suspended, and that developmental prolongation could be divided into two groups, which are cold torpor and diapause (Ewert, 1991).

1.1 Cold torpor and diapause

As mentioned above, developmental dormancy in developing embryos can be divided into two groups, such as cold torpor and diapause. Cold torpor is a periodic developmental layoff in the embryo of which the development has already begun (Ruf and Geiser, 2014). Cold torpor occurs in many species to reduce their energy expenditure during periods of stressful events such as cold exposure (Geiser, 2004). In such species, the brooding of eggs start when the last egg in the clutch is laid, therefore the first egg to be laid in the clutch will experience the longest exposure to cold torpor (Winkler and Walters, 1983). Cold torpor is known to occur most abundantly in avian and reptilian species during the clutch period. In reptilian species, the embryos are able to tolerate low temperature exposure up to 20°C

during embryonic development and able continue normal development to hatching (Yntema, 1960).

On the other hand, the other type of developmental suspension in the embryo, which is embryonic diapause, refers to a developmental arrest in normal organisms in response to unfavorable environmental conditions (Renfree and Shaw, 2000). Diapause occurs in a various scope of animals including early embryos of mammals, whose blastocyst undergo a period of totally quiescent or slow grow rate in the uterus. In mammalian species, the intrauterine implantation is suspended by the stimulation of estrogen and progestins on the dormant blastocyst can control the mitotic arrest of a diapausing embryo (Lopes et al., 2004). The entrance into diapause is triggered by the signals such as the absence of nutrition or photoperiod, and usually requires conformational change that increases resistance to extreme environments, which could be fatal in the normal status (Schiesari and O'Connor, 2013).

1.2 Embryonic dormancy in various species

Embryonic dormancy occurs in many species, but the exact mechanisms which control the process is diverse among different species. In general, when animal enter torpor or hibernation period, the metabolic rate and body temperature drops significantly to conserve the expenditure of energy during the dormant phase (Geiser, 2004). Early studies on the embryonic dormancy of animals were conducted on the oviparous species, because the dormant period of the embryos was most evident. For example, in reptilian species, such as turtles,

fresh eggs of *Chrysemys picta* turtles are able to withstand freezing temperatures for up to one month with normal development at optimal temperature (Cunningham, 1922). At the time of research the exact molecular mechanisms relying the strong tolerance of turtle emrbyos were vague. However, advanced studies show that hypoxia is the triggering cue of embryonic dormancy in reptilian species, and upregulation of insulin-like growth factor binding protein (IGFBP-1), which is bound to insulin-like growth factors (IGFs) during embryonic hypoxia cause the embryo to enter dormancy (Rafferty and Reina, 2012). Embryonic dormancy also occur in lower species such as grasshopper *Melanopus* of which is known to form a fibrous membrane around the egg to protect the it from possible threats to the egg (Shutts, 1952). The embryo is covered with the fibrous membrane until hatching, and secretion of the hatching enzyme degrades the membrane enabling the hatching of grasshopper hatchling. In the annual killfish, after oviposition, the embryo enters into a state of metabolic dormancy, which enables it to survive under complete absence of oxygen (Podrabsky and Culpepper, 2012). When the annual killfish embryo enters the dormant state under anoxic environments, the ATP content severly reduces and as a result most cells are arrested in the G_1/G_0 phase of the cell cycle. Similar to the killfish, embryos of crustacean *Artemia francisca,* which is known as brine shrimp, are able to endure extreme anoxia or hypothermia. Upon the shift to an unfavorable (freezing or anoxic) environment, the *Artemia* emrbyos form a cyst, which is produced oviparously with rigid, chitinous shell (Qiu et al., 2007). The encysted embryo show unique gene expression, and mainly the LEA gene contribute the tolerance of

the embryo against external stress factors (Toxopeus et al., 2014). Many embryonically dormant or diapausing species are studied to elucidate the physiological and molecular mechanism of entry and escape of dormancy, but the exact phenomenon occurring in the early avian embryo during embryonic dormancy is yet to be elucidated.

1.3 Embryonic dormancy in avian species

Cold torpor after oviposition is a natural occurrence in the avian embryos, due to its nature of brooding several eggs in a single clutch at once (Winkler and Walters, 1983). All avian embryos ovipositioned are advanced in development to a blastoderm or less, therefore the earlier the egg is ovipositioned in the clutch, the longer it undergoes cold torpor during development. Therefore cold torpor is common in diverse species of birds, and particularly in unincubated eggs (Webb, 1987). Not only the temperature is a cue to entry of dormancy in avian species, but also the availability of oxygen is a cue to dormancy. When the early avian embryo is inside the oviduct, the shell is deposited around the embryo in the shell gland, and the prevention of the embryo from abundant oxygen consumption marks the entry of dormancy (Rafferty and Reina, 2012). Once the incubation begins, the embryos develop without suspension unless the embryo is damaged or been too long in the dormant state. Similar to the dormant period after oviposition, the avian embryo also endures some extent of temperature decline during the incubation period, which varies among species, and extreme species can hold development for several hours to days under unfavorably low temperatures (Cooper and Voss, 2013).

The ability to continue normal development after dormancy in avian species is important not only in the wild avian species, but also important in the domesticated avian species due to their production of eggs for industrial purposes. In the poultry industry, egg storage is a common and important practice for the hatchery of healthy chicks (Gomez-de-Travecedo et al., 2014). In general, the chicken egg can be stored for up to 2 weeks at approximately 15 to 20°C with minimum affect on the hatchability of the chicks (Fasenko, 2007). After oviposition, the internal temperature of the egg declines drastically, which reaches the hen house ambient temperature within several hours (Patterson et al., 2008). Once the egg is ovipositioned and internal temperature drop, the chick embryo arrests development and enters embryonic dormancy or cold torpor until it meets optimal temperature for development. There have been many practices to improve the hatchability of chicken embryos after long period of storage, such as pre-incubation warming or injection of biological buffers. The pre-incubation warming method requires brief incubation before the storage of eggs, and it is known to increase the hatchability when storage time is prolonged, but does not affect the quality of the chick (Dymond et al., 2013; Reijrink et al., 2010). The other method, which is to inject biological buffers into the pre-storaged eggs, is an approach to reduce the physiological changes in the egg that affects the embryo quality. This method utilizes the *in ovo* injection of slightly acidic buffer into the egg, to prevent the albumen pH increase during storage, and show improved hatchability and extends storage period of chicken eggs (Akhlaghi et al., 2013).

In addition to the physiological changes that occurs in the early chicken blastoderm, the changes in the molecular level of the embryo is important in the endurance of external stress during storage. There is still handful of information about the exact molecular mechanisms that occur in the dormant chicken embryo. First of all, it is known that the amount of apoptotic cells increase during the storage of chicken blastoderm (Hamidu et al., 2010). Interestingly, this study demonstrated that the amount of necrotic cells is abundant in the unstored avian blastoderm, where it decreases after storage. Another study intentionally induced apoptosis in the avian blastoderm cells to measure the resistance of the blastodermal cells to stress. In this study, the expression of apoptotic cell death gene in blastoderm cells were observed after induction of apoptosis by various stimulants such as etoposide, cytotoxic drugs or heat shock. The results show that the blastodermal cells were highly engaged in the apoptotic process, but did not progress further until cell death due to the continuous expression of anti-apoptotic genes namely bcl-2, bcl-X_L and hsp70. The results strongly indicate that the avian blastoderm has ability to protect itself against external stress factors, by upregulation of genes that antagonize cell death (Bloom et al., 1998). As previously demonstrated, the avian blastoderm contains ability to stand against the threats of cell death under stressful events, and at the same time extension of the duration of storage or stress causes increase of cell death. However, although the molecular phenomenon that occurs in the avian embryo during dormancy is known, the exact mechanisms and reason of the strong resistance to stress is still unknown. To understand the ability of the avian embryo to withstand the stress

during storage period, more extensive research on the nature of the blastoderm should be conducted.

2. Survival mechanisms at the cellular level

As soon as the avian embryo is ovipositioned, the embryo is under acute cold stress, which has negative effect on survival at the embryonic level as well as the cellular level (Patterson et al., 2008). Both acute and chronic stress threatens the cell survival, and rapid adaptation or resistance should be taken to maximize the probability of cell survival (de Nadal et al., 2011). There are multiple cellular stress adaptation mechanisms, which include the regulation of gene expression or protein signaling. First of all, the cellular stress adaptation mechanisms controlled by the regulation of gene expression mostly involves the heat shock protein family genes. The heat shock protein (HSP) family genes are extensively conserved in many species, and are expressed in reaction to various types of stress. The HSPs are expressed in order to protect the cell against aggregation and misfolding of protein under various types of stress (Richter et al., 2010). Most HSP gene expression is controlled by the heat shock factor (HSF), which is a transcription factor that acts as a dimer on the upstream of HSP genes (Akerfelt et al., 2010). Under heat shock or osmotic stress, the HSP genes are upregulated in few minutes to hours to maintain homeostasis in the cell. On the other hand, the cellular stress levels can be controlled with protein signaling, especially the stress-activated protein kinase (SAPK) signaling. SAPK signaling pathways include the ERK, p38 and JNK pathways, which

will be discussed in more detail. Each pathway utilize phosphorylation of kinases to convey stress signals, and as same as the HSP's reaction to stress, SAPK signaling can occur in few minutes to hours in response to stress, which enables immediate relief of imbalance in cellular homeostasis (Bode and Dong, 2007).

Although the HSPs and SAPK signaling are the hallmark of cellular stress, there are many other genes and cellular processes that are regulated in response to stress. For example, the zebra fish larvae experience both hypoxia and hypothermia after oviposition. To study the genes and pathways that are involved in each stimulus, the transcriptional events were characterized by RNA sequencing. As a result, it was shown that similar gene expression patterns and molecular pathways were activated under hypoxic and hypothermic conditions, including the major reduction of metabolic processing related genes. This study demonstrated that there is plethora of genes that are controlled in response to stress, which under normal conditions involved in other cellular processes, and multiple pathways are controlled by the change in external stress (Long et al., 2015).

3. Endoplasmic reticulum (ER) stress

The endoplasmic reticulum (ER) is the central organelle in the secretory pathway, which is responsible for the protein translocation, folding and post-translational modifications that allow the transport of the proteins to the Golgi apparatus, and finally secretion to the places of the need of specific proteins (Sano and Reed, 2013). Most of the eukaryotic cells have evolved to utilize a conserved mechanism in the

adaptation of stress, especially in protecting the protein integrity. ER stress is one of the major protein protection mechanisms against stress, which enables the normal cellular function after an acute exposure to stress. The initiation of ER stress marks the unfolding and aggregation of cellular proteins, and the ER stress mechanism enables the adaptive response in the cell (Xu et al., 2005). The activation of ER stress is mediated by the unfolded protein response (UPR), which performs stress-buffering activity for the ER.

3.1 Unfolded protein response (UPR)

The protein-folding stress at the ER is buffered by the activation of unfolded protein response (UPR), a homeostatic recovery network that recovers the ER function during stress (Cao and Kaufman, 2012). The protein secretory machinery in the ER is continuously under stress, because of its high demand for protein processing. The adaptive mechanism in response to the high protein folding demand in the ER is the UPR, which is a process that updates the protein-folding status in the ER lumen, and transduces it to the nucleus where it reacts by decreasing the amount of protein production or eliminates damaged cells by apoptosis (Woehlbier and Hetz, 2011). Under stressful conditions, the UPR reduces unfolded protein load into the ER through various mechanisms; however, when the ER stress is not reduced the UPR initiates apoptosis for the clearance of cells (Gardner et al., 2013). The UPR can be divided into two mechanisms, which include the adaptive mechanism and chronic or apoptotic mechanism. The adaptive UPR mechanism was initially characterized by the

Saccharomyces cervisiae, which indicated that the demand for large gene expression in this organism caused an unknown mechanisms to cope with the excessive demand for protein secretion (Ron and Walter, 2007). Proceedings in the research indicated that the UPR has evolved into a network of mechanisms that involve multiple cellular responses that decide the cell fate under ER stress. The adaptive response is mediated by the attenuation of translation or enhancement of protein quality control, folding or redox reactions. The adaptive response to UPR usually involves short, acute or mild level of stress, which progress to the late or apoptotic phase of UPR when level and duration of stress increases (Hetz, 2012).

The late phase or apoptotic phase of UPR includes the many cellular death signals and genes. As the level of stress increases, the adaptive response of UPR is changed to the apoptosis phase by the activation of caspase family genes (Tabas and Ron, 2011). Also the CHOP gene is known as a apoptotic mediator in the UPR signaling, which directly inhibits the Bcl-2 anti-apoptotic genes, and upregulate the apoptotic signals (Poone et al., 2015). Once the apoptotic genes are active in the UPR signaling response, the apoptosis phase is active, and the cell is programmed to death.

3.2 Effector pathways in ER stress

The ER stress is a common feature in many organisms affecting the function of the protein secretion machinery. The ER stress-signaling pathway is conducted by three different effectors on the ER lumen, which are IRE1, PERK and ATF6. Each of the effector

balances the capacity for protein maturation in the ER stress processing, though each utilizes different signaling proteins for a similar outcome, which is apoptosis as the final step (Urra et al., 2013). In general, the activation of ER stress effectors is achieved by the UPR, especially the binding of unfolded protein and chaperone complex to the outer structure of the effector (Woehlbier and Hetz, 2011). Once the unfolded protein-chaperone complex is bound to the effector, the effector activates the ER stress-signaling network via phosphorylation or cleavage of downstream molecules.

First of all, inositol-requiring enzyme 1, also known as IRE1, is a ER transmembrane sensor that can initiate apoptosis through the decay of anti-apoptotic miRNA or conversely promote survival in some organisms. The IRE1 arm directed towards the cytosol is conserved in most eukaryotes, and it is activated by the binding of the chaperone complex, which phosphorylates the opposite arm and prepares it for activity. IRE1 is known to have three different activities, which include 1) the initiation of JNK signaling pathway, 2) the splicing of bZIP for export into the nucleus, and 3) the mRNA decay for deduction of protein loading into the ER (Chen and Brandizzi, 2013). JNK signaling activation occurs by the phosphorylation of the map kinase (MAPK) JNK, which through signal transduction mediates apoptosis (Cao and Kaufman, 2012). Recently it was found that the JNK signaling pathway is selectively activated by mTORC, which causes apoptosis through the suppression of Akt and consequent induction of the IRE1-JNK pathway (Kato et al., 2012). In case of the bZIP splicing, IRE1 splices the transcription factor Xbp-1 and bZIP60 mRNA, then the spliced factors enter the nucleus to regulate the UPR target genes, ultimately

reducing the protein load into the ER promoting the survival of the cell. This process is similar to the mRNA decay, which occurs in the ER translocating proteins, by direct degradation of mRNA thus preventing the secretory pathway of proteins. On the other hand, if the level or duration of stress increases, the cell undergo apoptosis, which is triggered by IRE1 by selective production of miRNAs targeting caspase-2, thus promoting the release of cytochrome-c, which induces cell death (Chen and Brandizzi, 2013).

The other type of ER stress effector is PRKR-like endoplasmic reticulum kinase (PERK), which is a Ser/Thr protein kinase that shares the catalytic domain of the substantial homology to other eIF2α family kinases (Harding et al., 1999). The importance of IRE1 in ER stress mediation is proved in $IRE1^{-/-}$ cells, which has lost the ability to activate eIF2α (Scheuner et al., 2001). The activation of PERK by chaperones induces the auto-phosphorylation of the kinase domain, and this phosphorylates and inactivates eIF2α, reducing the mRNA translation and protein load into the ER (Kim et al., 2008). However, the transcription factor ATF4 is rather up-regulated with the PERK phosphorylation, because of its role in regulating the promoters of several genes in the UPR. For example, the molecular chaperone GRP78 and GRP94 gene promoters are regulated by ATF4, and this implicates the accessibility of ATF4 on the ER stress and UPR response genes (Harding et al., 2003).

The third type of ER stress effector is ATF6, which is a bZIP family transcription factor, similar to ATF4. The ATF6 has a rather unique mechanism in action, because it involves the GRP78 secretion to the Golgi body, where it is cleaved and processed then exported

into the nucleus for regulation of ER stress target genes (Ye et al., 2000). It is also known that ATF6 has resistance to hypothermic stress, although the exact genes that regulate the process are not known (Thuerauf et al., 2007). Regulator of calcineurin (RCAN1) is one of the candidate gene involved in the hypothermic resistance process of ATF6, which is explained by the inhibiting activity of calineurin whose substrates are pro-apoptotic genes, suggesting that the ATF6 hypothermia resistance is achieved through the inhibition of pro-apoptotic genes (Belmont et al., 2008).

3.3 ER stress and SAPK signaling

As discussed above, the ER stress signaling mechanism is related with the activation of SAPK signaling through the phosphorylation of the IRE1 effector on the ER lumen (Cao and Kaufman, 2012). Commonly the UPR is deeply related with the cellular stress, and this initiates the phosphorylation of JNK or p38 by the action of IRE1. These kinases are also responsible for the activation of the transcription factor nuclear factor-κB (NF-κB), which indicates that the ER stress mechanism is related with the cellular signaling process (Kaneko et al., 2003). IRE1 has functions in controlling the initiation of cellular MAPK signaling, for example, the apoptosis signal-regulating kinase 1, also known as ASK1, is reported to be related with JNK activation. This was found by utilizing $IRE1^{-/-}$ fibroblasts showing that the JNK activation by ER stress was impaired. It also explains that the IRE1 is bound to TRAF2, which is an adaptor protein that induces the activation of JNK signaling, and this was also shown by dominant

negative TRAF2 cells that had retarded ability to activate JNK by IRE1 (Urano et al., 2000). These results indicate that the JNK activation in response to ER stress is an important determinant in the cell death decision.

3.4 Mechanisms involved with apoptosis

The transcription factor C/EBP homologous protein (CHOP, DDIT or GADD153) is the downstream component of the three different effectors of ER stress pathways, which is involved in the induction of apoptotic genes as the final step of ER stress (Ma et al., 2002). The CHOP gene promoter has binding sites for major inducers of the UPR, such as ATF4 or ATF6, which were proved to be responsible for the upregulation of CHOP during the ER stress period. CHOP is also related to the p38 SAPK signaling pathway, utilizing the ASK1 protein as the mediator to control the activation of signaling (Wang and Ron, 1996). The upregulation of CHOP is involved in the apoptotic activity, which is controlled by the Bcl-2 anti-apoptotic protein (McCullough et al., 2001). Recent studies also show that CHOP is related to Ero-1 in ER stress under the hypothermic influence. It is suggested that the hypothermia suppress the ER stress induced apoptosis, and also the expression of CHOP (Poone et al., 2015).

4. Stress activated protein kinase (SAPK) signaling

The stress activated protein kinase (SAPK) signaling, is an evolutionally conserved cellular signaling mechanism utilizing the

mitogen-activated protein kinases (MAPK), for the conductance of information about the external and internal stress to the cell. MAPK is a family of proteins that transduce environmental and developmental signals into cellular responses such as differentiation, proliferation or apoptosis (Cowan, 2003). The MAPK action by the addition and subtraction of phosphor groups on the phosphorylation sites, which enables fast reaction against change of cellular status. The MAPK signaling pathways include three action molecules, which are MAP3K, MAP2K and MAPK. Phosphorylation of the molecules is conducted downstream from the MAP3K, which finally activates the transcription factor that is responsible for cellular processes (Chang and Karin, 2001). The SAPK signaling pathway can be divided into three subgroups depending on the MAPK molecule, which are extra cellular signal-regulated kinases (ERK), c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK), and p38 MAPK. Especially the activation of the JNK pathway is induced by a variety of stress, which includes heat shock, metabolic stress or osmotic stress (Gehart et al., 2010).

4.1 JNK signaling pathway

The JNK signaling pathway is the representative and most studied signaling pathway that is activated in response to cellular stress. JNK has three types of genes, including JNK1, JNK2 or JNK3 that each can be alternatively splice to give rise to at least 10 isoforms (Gupta et al., 1996). JNK1 and JNK2 are expressed universally in most cells, while JNK3 is specifically expressed in the brain (Bode and Dong,

2007). In addition to the cellular stress recognition, the JNK pathway also acts as a pathway for cellular proliferation, differentiation, survival and migration (Wagner and Nebreda, 2009). Previous studies in mice and human has elucidated the functions of JNK in various processes, for example, a study using JNK1 and JNK2 double negative knockout mice have shown that the JNK signaling is important in the cytochrome c release and apoptosome activation, indicating that JNK is crucial for the activation and execution of apoptosis (Tournier et al., 2000). Especially JNK is responsible for the regulation of transcription factors such as c-Jun, ATF2, Elk1 and p53, which are mostly related to cellular processes such as cell death or apoptosis. Therefore, studies using the JNK knockout mice indicate that JNK signaling is closely related to the cellular apoptotic process, by controlling the transcription factors that are responsible for cellular death processing.

In addition to the regulation of transcription factors for cellular process, JNK is able to suppress the cellular transcription machinery to defend from environmental stress, which is achieved by the assembly of stress granules (SGs). Stress granules are multimolecular aggregates that are composed of untranslated mRNAs to prevent the accumulation of misfolded proteins in the ER (Anderson and Kedersha, 2008). It is found that the stress granule formation is related to the JNK signaling by the mediating molecule RACK1, which binds to the MAP3K and facilitates the activation by environmental stress (Meyerowitz et al., 2011).

In addition to the functions of JNK in normal cells, its function in cancerous cells are also identified by the abnormal ability of JNK to phosphorylated c-Jun and AP-1 transcription factors, which is the

dimeric family of transcription factors of Jun, Fos protein families, impairing the tumor suppressive functions in the cell (Eferl and Wagner, 2003). The JNK signaling pathway regulates multiple genes that contain an AP-1 binding site, including genes that control the cell cycle, implying the role of JNK in various other cellular processes that can be related with the cellular malfunction. Although many are known about the function of JNK signaling, the exact molecular network is yet elucidated, thus deeper investigation of the individual molecular interaction and network is necessary. To understand the signaling signatures of SAPK pathway, it is crucial to know the functions and molecular network of other SAPK pathways, because the SAPK signaling pathway is mostly the crosstalk between several signaling pathways.

4.2 p38 signaling pathway

The p38 MAPK signaling was first identified as a mammalian homologue to the yeast protein Hog1, and is a group of MAP kinases that serve a role in numerous biological processes, including the cellular response to stress (Han et al., 1994). There are multiple studies that suggest the p38 pathway is related to inflammation, cell cycle, cell death, development or cell differentiation (Zarubin and Jiahuai, 2005). As part of the SAPK signaling molecule, p38 has roles in the induction of cell death or apoptosis, which is controlled by four MAPK genes that encode for p38. Those four genes have alternatively spliced forms, increasing the diversity in function of the p38 molecule (Sanz et al., 2000). The p38 MAPKs are activated by the upstream

MAP3K MKK family, which is similar to the JNK pathway. The activation is achieved by phosphorylation of the p38 by MKK proteins or also by autophosphorylation of p38 (Mittelstadt et al., 2005). Once the p38 signaling pathway is active, numerous cellular processes occur in response to the signal transduction.

The cell cycle can be regulated by the p38 signaling pathway in both G_1/S and G_2/M transitions, generally through the control of cyclins and cyclin-dependent kinases (CDK) that are responsible for the progression to the next cell phase of cell cycle. In most case, the activation of p38 signaling pathway negatively affects the cell cycle, by arrest at several checkpoints of cell cycle, which includes the regulation of tumor suppressor p53 (Rincon, 2009). It is also found in the p38 MAPK knockout mice that the cell cycle was arrest by the mitotic arrest at G_2/M phase (Takenaka et al., 1998). The activity of p38 is also related to the cell proliferation, which is in most case down-regulated by p38 signaling (Hui et al., 2007). One of the most important functions of the p38 signaling pathway is the induction apoptosis in response to cellular stress, and the mechanisms include transcriptional and post-transcriptional processes depending on the type of apoptotic stimuli. For example, for stress induced by reactive oxygen species (ROS) activates the p38 signaling to initiate the apoptotic pathway and enables the suppression of tumor growth inhibition in cancer cells (Dolado et al., 2007). However, the p38 signaling is also known to have both anti- and pro- apoptotic functions, which can be a double-sided sword for the cellular survival. The complex signaling transduction cascade enables the tight regulation of apoptosis with other multiple roles in cellular responses. Collectively, the previous

studies on the p38 signaling pathway shows that it is essential for many cellular process, especially for cell death and apoptosis, and could encourage or discourage the cellular death.

4.3 SAPK activation in hypothermic situations

The JNK signaling pathway and p38 signaling pathway shares multiple upstream regulators that is differentially expressed depending on the type of stress or response of the cell. Especially when the avian embryo is ovipositioned, it undergoes hypothermic stress, which is caused by the different temperature from the oviduct. Therefore, low temperature stress is a key factor that contributes to the activation of stress activated signaling in the avian blastoderm after oviposition. The SAPK signaling is known to be up-regulated in response to various stress including the hypothermic stress, which is demonstrated by multiple studies. For example, the study using human umbilical vein endothelial cells (HUVECs) has shown that they had activated JNK and p38 signaling throughout the exposure to hypothermic stress, and this was related to the viability of the cells (Roberts et al., 2002). Also the acute cold exposure of the rat brain cells shown enhanced spatial memory along with the increased activity of SAPK signaling, especially the p38 and JNK signaling cascade. It is argued that the level of JNK and p38 phosphorylation has significantly increased after the cold exposure, but drastically drops when the brain cells were rewarmed to normal temperature. Even after the exposure to cold stress, the rats were able to perform tasks identically as the normal untreated rats. The results indicate that the SAPK signaling pathway provides

significant degree of protection or defense of the rat brain cells during hypothermia (Zheng et al., 2008). The effect of SAPK signaling is not only limited to the somatic cells, but also has effect in the germ cells. In the study using mouse testicular cells and spermatocytes, it was shown that the cold-inducible RNA-binding protein (CIRP) activated the MAPK signaling and consequently impaired the spermatogenic functions. CIRP is an RNA-binding protein that is expressed during the normal development of testes, and mostly down-regulated when the testes are under heat stress. The researchers claim that the SAPK signaling was down-regulated after the down-regulation of CIRP, which is induced by cold stress. Therefore, it could be suggested that the SAPK signaling pathways are related to the regulation of cold-inducible genes and mechanisms executing the cellular responses in response to hypothermic stress (Xia et al., 2012). Extensive research about the effect of hypothermia on cells is performed in purpose to understand the cellular mechanisms that occur during transplantation of organs, be cause it is important to preserve it in a cold temperature to prevent damage in the organ. Several studies suggest that the SAPK signaling is active throughout the exposure to hypothermia, in various types of organs that are transplanted (Casiraghi et al., 2009; Crenesse et al., 2000; Lotocki et al., 2006). It is also known that the hypothermia induces apoptosis through the activation of SAPK signaling, especially by the upregulation of downstream transcription factor c-Jun. During hypothermia, the SAPK signaling was stronger than normothermia, and it upregulated the activity of TNF-α consequently increasing the amount of apoptotic cells. Inhibition of the MAPK by chemical inhibitors decreased the amount of cleaved caspase-3, suggesting that the

SAPK signaling is able to reduce the apoptotic activity (Yang et al., 2010). In conclusion, the SAPK signaling is deeply related with the change of environmental temperature, especially against cold stress the signaling activity is dynamically regulated to defend against negative cellular events such as apoptosis.

5. Apoptotic cell death

Apoptosis, which is also known as a type of programmed cell death (PCD), is characterized by the perturbations of the internal cellular structure, which does not only contribute to cell death, but also prepares the clearance of abnormal cells by the phagocytes and prevent unnecessary immune reactions (Taylor et al., 2008). PCD is a unique cellular process, because it is a tightly controlled mechanism of multiple cell death related genes, and failure in the tight regulation of those genes causes various types of pathologies or immature cell death signaling. Apoptosis is marked by the phenotypical and genetic change in the cell, and both change are important in the detection of apoptosis. Phenotypically an apoptotic cell is distinguished by the plasma membrane blebbing, chromatin condensation and margination and formation of apoptotic bodies (Krysko et al., 2008). At the gene expression level, apoptotic cells exhibit high-level expression of caspases and cytochrome c release (Sato et al., 2008). Apoptosis is a highly conserved cell death mechanism across various organisms, which mostly employs the highly conserved caspase expression for regulation of the signals, and this enables the highly-conserved

initiation and execution mechanisms across various organisms (Huang et al., 2000).

5.1 Mechanisms of apoptosis

The evolutional conservation of apoptosis is most prominent in the caspase family of genes (Huang et al., 2000). All known apoptotic signals consists of the molecular signaling of caspases. To date, there are two main apoptotic initiation pathways, which are the extrinsic and intrinsic pathway. The extrinsic pathway or death receptor pathway involves the binding of extracellular death ligands to the transmembrane death receptors, which activates the adaptor proteins such as fas-associated death domain protein (FADD), and this stimulates the cascade of caspases (Ashkenazi and Salvesen, 2014). Caspases are a family of endoproteases that control the cellular networks related to inflammation and cell death. The death receptors involved in this pathway mainly involves the tumor necrosis factor (TNF) receptor gene family, and the TNF ligand binding causes the recruitment of FADD or TRADD proteins (Locksley et al., 2001). In the extrinsic pathway, the activation of caspase8 marks the execution of apoptosis, thus once caspase8 is provoked, the execution of apoptosis is initiated. The activation of caspase8 is achieved by the cleavage of procaspase8 into an active caspase8 form (Lin et al., 1999). The other activation pathway is the intrinsic pathway or mitochondrial pathway, which is initiated by a non-receptor-mediated stimulus that produce intracellular death signals, and those events are involved with the mitochondria. The intrinsic pathway of apoptosis is initiated by various

ranges of stresses, which include toxins, hypoxia and hypothermia. Those types of stress causes the imbalance in the homeostasis of the mitochondrial membrane, and this results in the opening of the mitochondrial permeability transition pore (MPT), and the loss of membrane potential leads to the activation of pro-apoptotic genes (Locksley et al., 2001). The initial proteins that are controlled in reaction to the MPT pore opening include cytochrome *c*, DIABLO protein, and caspase -8, -9, -10 (Elmore, 2007). The activation of initiator caspases is generally achieved by the dimerization of caspase monomers, rather than cleavage (Boatright et al., 2003; Chang et al., 2003). The dimerization of caspase monomers induces the catalysis of the monomers, which stabilizes the activation ability of the dimers. Once the initiation caspases are dimerized and active, those proteins recruit the aggregation of caspase9, which marks the execution of apoptosis (Pop et al., 2006).

Both extrinsic and intrinsic pathway of apoptosis initiation ends with the promotion of the execution pathway, which is mainly transduced with different types of caspases. The caspase family genes are important in both initiation and execution of apoptosis, and the initiator caspases include caspase -8, -10, -9, -2, on the other hand the executioner caspases include caspase-3,-6,-7 (McIlwain et al., 2013). The correct regulation of caspases is important in cellular death mechanisms, because it is important to avoid unnecessary inflammation or damage to the surrounding cells. As a mechanism to protect from unnecessary activation of executioner caspases, they are produced as a procaspase that must be cleaved by the initiator caspases, and this provokes conformational change in the active

binding sites of the executioner caspases (Riedl and Shi, 2004). The apoptotic execution phase is marked by the upregulation of caspase3 by the initiator caspases, and caspase3 is considered the most important type of caspase among the executioners. The reason is because of the ability of caspase3 to specifically activate the endonuclease caspase-activated DNase (CAD). The majority of apoptotic cells contain activated CAD, which promotes the degradation of DNA and condensation of the chromatin, displaying the hallmarks of apoptosis (Enari et al., 1998). After the DNA breakage and chromatin condensation, the last step of apoptosis is the phagocytic uptake of the apoptotic cells. The imbalance in cellular membrane homeostasis exposes the phosphatidylserine on the surface of the apoptotic cells, and the phagocytes recognize the apoptotic cells that represent those markers on the surface, processing the engulfment (Fadok et al., 1998).

5.2 Stress mediated activation of apoptosis

Apoptosis is induced by various cellular signals including heat shock, osmotic stress, free radicals or chemicals. There are many molecular signaling pathways that are activated in response to cellular stress and death, and most of the mechanisms involve apoptosis as the final step that leads to cellular death.

One of the cellular stress adaptation mechanisms involves the action of heat shock proteins (HSPs). The HSPs are molecular chaperones that are produced in response to different types of stress-induced cell damage. Under normal conditions, the HSPs functions in multiple normal cellular roles, though under stressful situations, the

HSPs act to protect the native proteins by preventing the misfolding or degradation (Richter et al., 2010). HSPs are known to also regulate the intrinsic or mitochondrial pathway of apoptosis by overexpression of various protective HSPs in against to stressful stimulus (Takayama et al., 2003). For example, the overexpression of hsp27 increases the resistance of cells to various apoptotic cues (Rogalla et al., 1999). Under stressful conditions, hsp27 binds to cytochrome c to interfere with the initiation of apoptosis. Also hsp70 is known to actively protect the cell from apoptotic progress, through the inhibition of caspase dependent events that occur in the later stages of apoptosis. Specifically, it prevents the binding and activation of caspase3, which inhibits the downstream events of apoptosis (Jäättelä et al., 1998). It is also known that the HSPs are involved in the extrinsic pathway in the TNF family receptor mediated mechanism through the binding of hsp70 to the TNF receptor. This blockage of apoptosis via HSP is related to the JNK signaling, which is a representative stress-activated protein kinase (SAPK) signaling (Gabai et al., 2000).

In addition to the caspase-mediated activation of apoptosis, under the influence of chronic stress, the cell activates stress-activated protein kinases (SAPKs) that are responsible for the stimulation of the apoptotic pathway. SAPK signaling includes ERK, p38 and JNK signaling, which are all activated by external stress. Apoptosis is closely related to the three signaling pathways, because the final phenomenon that occurs in those pathways is apoptosis (Cho and Choi, 2002). In most SAPK pathways, the MAPK phosphorylates various substrate proteins, which includes transcription factors as c-Jun, ATF2, Elk1 or p53. As an example, there have been studies to

elucidate the functions of JNK in apoptosis through mutant knockout mice. From those studies, it was shown that JNK double knockout mice fibroblast were impaired in the activation of cytochrome c and formation of apoptosome by UV induction of stress signals, therefore had lower apoptosis rate. This indicates that the mitochondrial pathway of apoptosis induction is influenced by the JNK signaling (Tournier et al., 2000). Also in the JNK knockout in mice caused lower stress-induced apoptosis in the hippocampal neurons, indicating the function of JNK in the programmed cell death in the neuronal cells (Whitmarsh et al., 2001). Recent study also demonstrated that inhibition of JNK signaling diminishes the early signs of apoptosis such as phosphatidylserine representation on the cellular membrane, but it did not affect the late apoptotic status as caspase3 activation, hence showing that JNK signaling has close related in activating the initiating signals in apoptosis (Krilleke et al., 2003). Collectively, the studies using JNK knockout mice show the importance in the JNK signaling pathway in the induction of apoptosis and the indispensable role in cell death.

5.3 Reversal of apoptosis

It was understood that apoptosis is an irreversible death pathway since the apoptotic mechanisms were elucidated. However, recent studies demonstrate that apoptosis can be reversed if the apoptotic stimuli is removed and cell is taken back to the normal growth conditions. To understand the reversibility of apoptosis, it is important to understand the different status of apoptosis, which can be divided

into the early and late phase. The early phase of apoptosis is characterized by the presentation of phosphatidylserine on the leaflet of the cell membrane, and also activation of initiator caspases such as caspase -9, -8, -10. In the early apoptotic phase, the executioner caspases are yet activated, and there is also no damage to the chromatin or DNA. During the late apoptotic phase, the initiator caspases dimerize and activates the executioner caspases, which cause condensation and breakage to the chromatin and DNA (Poon et al., 2010). Once the genetic information and cellular membrane is damaged, the apoptosis is not reversible.

During the early phase of apoptosis the damage to the cell is very low, and removal of stressor can return the cell into the normal status. In the study of Tang et al., various types of cell lines were used to observe the ability to return to normal state after induction of apoptosis. Short exposure to different types of apoptotic stimuli as UV or inducer chemicals caused induction of early apoptosis, which was demonstrated by the presentation of phosphatidylserine on the outer cell membrane without any chromatin margination. However, after the short exposure, when the apoptotic stimuli were washed off, the cells were able to return back to the normal state, which was indicated by the lack of phosphatidylserine detection of the leaflet of the membrane, or lack of chromatin margination. The results indicate that the dying cells can recover from apoptotic execution stage after the removal of apoptotic stimuli (Tang et al., 2009). They also have confirmed the reversible apoptosis occur in cancer cells, which explains the recurrence of cancer cell after chemotherapy (Tang et al., 2009). Tang et al. have nominate this mechanism “anastasis”, which means “rising

to life" in Greek. The potential benefit of the reversible apoptosis is the possibility of preserving the cells that are irreplaceable during the stressful situations.

The previous results regarding the reversible ability of apoptotic cells can be hopefully applied to *in vivo* tissues or cells to understand the stress resistance in various organisms, specifically in the avian embryos during dormancy. To understand the avian embryo's nature in stress resistance, the ability to endure and reverse apoptosis should be carefully investigated, and in addition to apoptosis, mechanisms related to the stress response should be investigated as well.

6. Nucleotide metabolism and cell cycle

The synthesis of nucleotides is one of the most basic and fundamental processes that occur in the cellular level, because it is required for the synthesis and breakdown of nucleic acids. Nucleotide biosynthesis is performed by purine and pyrimidine, which are the basic components of the biosynthetic process. Owing to the fundamentality of this process, the enzymes that participate in the biosynthetic process of purine and pyrimidine are known to be housekeeping enzymes that are responsible for the basic cellular activities (Moffatt and Ashihara, 2002). Since the nucleotides are the elemental building blocks of the genetic information, the metabolism and breakdown of nucleotides are closely related to the cell cycle, since it is characterized by the division and proliferation of cells and furthermore division of genetic information.

6.1 Nucleotide metabolism

Purine and pyrimidine molecules are the basic building blocks of the nucleotides, and major carriers of energy. The purine and pyrimidine biosynthesis and metabolism is one of the most fundamental and conserved mechanisms among organisms, because of its importance in the composition of nucleic acids. The nucleotide metabolism is conducted by several key enzymes such as PRPP (phosphoribosyl-1-pyrophosphate), which uses molecules as CO_2 and tetrahydrofolate to convert the purine and pyrimidines into nucleotides. The nucleotide biosynthesis is achieved in two different pathways, which are the *de novo* and salvage pathways. First of all, the *de novo* pathway is a mechanism that newly builds nucleotides from purine and pyrimidine building blocks utilizing the PRPP enzyme (Henderson and Paterson, 2014). The final product of the de novo pathway is AMP (adenosine monophosphate) and GMP (guanosine monophosphate), which are further processed to be the basis of nucleotides. On the other hand, the salvage pathway, is the synthesis of nucleotides based on the cellular byproducts from metabolism, and compared to the *de novo* pathway that requires large amount of energy to create new molecules, the salvage pathway is energetically favorable, because it utilizes the catabolism of nucleic acids (Berens et al., 1995). The salvage pathway recycles the nucleotide triphosphates that are broke down by various metabolic reactions, and are rebuilt by the activity of enzymes such as phosphoribosyltransferase or adenosine phosphorylase (Traut, 1994). The metabolic process of nucleotides is very important for the unimpaired construction of nucleic acids, and the

dysfunction or damage during the synthetic process causes various diseases such as ischemia, due to the mutation in the enzymes that convert the purine or pyrimidine into higher order molecules (Nyhan, 2005). It is also known that the nucleotide metabolism is related to the cell cycle, especially during the S phase, as it is when the duplication of genetic information occurs. The nucleotide metabolism is known to be affected by the activity of MAPK signaling cascade, which controls the phosphorylation of CAD (carbamoyl-phosphate synthetase-aspartate carbamoyltransferase-dihydroorotase). CAD is the protein that initiates the purine and pyrimidine biosynthesis, and it is allosterically regulated by the MAPK signaling to initiate or block nucleotide synthesis. During the cell cycle progression, CAD activity fluctuates to regulate the amount of nucleotide biosynthesis and this is co-regulated with the cyclin expression throughout the cell cycle (Sigoillot et al., 2003).

6.2 Cell cycle

The cell cycle is one of the most important mechanisms in the biological processes, and is controlled by multiple pathways and proteins to prevent errors in the cycling. It is mainly related to the cell division, and DNA replication that includes the G_0, G_1, S, G_2 and M phase, and is progressed through several checkpoints that assure that the cell cycle is correctly performed. The cell cycle is managed and controlled by the complex of cyclins and cyclin-dependent kinases (CDK), and those molecules monitor the cell cycles throughout several checkpoints to progress or stop the cycle (Vermeulen et al., 2003).

Under stressed conditions, the cyclin and CDK complex or cell cycle regulation proteins such as p53 can induce the suspension or arrest in the cell cycle to protect the cell from malfunction (Vermeulen et al., 2003). One type of the stress that could arrest the cell cycle is the cold shock, and this is known to hinder the transition of G_2/M in various types of cultured cells (Rieder and Cole, 2014). However, the exact mechanism of how this occurs and which protein regulates it is still unexplored, and is only speculated that the cells exhibit synchrony under cold stress. One clue to this phenomenon is the drastic increase of p53 gene during hypothermia, which suggests that the cell cycle check point genes are active to control the progression of the cell cycle through G_2/M phase (Dulić et al., 1998). Another aspect is that under hypothermic situations, the cell cycle is affected by the signaling of SAPK pathways that resist the cell cycle progression into mitosis that is achieved by the inhibition of cyclin-CDK complex that regulates the transition into G_2/M phase (Pearce and Humphrey, 2001).

6.3 Cell cycle arrest and apoptosis

The response of cells to external stress can be managed by two important biological mechanisms, which are the SAPK pathways and cell cycle checkpoints. Especially the cell cycle checkpoints regulate the DNA integrity through the action of several checkpoint genes as p53, and the mutations to this gene is responsible for the generation of cancerous cells. The effect to of SAPK activation was demonstrated in the yeast, which has shown that the osmotic stress induced the extended G_2 phase (Alexander et al., 2001). Moreover, the role of p38

MAPK signaling cascade in the arrest of G_2/M is demonstrated by γ-irradiation on mammalian cells. It was shown that when the cells were irradiated by γ –ray, the MKK6 MAP3K was phosphorylated and responsible for the G_2 arrest (Wang et al., 2000). On the other hand, many studies argue that the G_2 arrest is related to the apoptotic progress of the stressed cell, due to the SAPK signaling and cell cycle checkpoint genes. Numerous studies were targeted to find the G_2/M cell cycle arrest methods in the cancer cells to discover anti-cancer therapeutics (Augustin et al., 2006; Ouyang et al., 2009; Vermeulen et al., 2003; Zhang et al., 2013). The results of the numerous studies indicated that the arrest in G_2/M phase is the main reason of the upregulation of apoptosis related genes and execution of the apoptosis.

CHAPTER 3

TRANSCRIPTOMIC ANALYSIS

OF THE DORMANT AVIAN EMBRYO

Introduction

At the cellular level, the most immediate change when external stress (temperature, oxygen availability) is present is the protein misfolding (Kozutsumi et al., 1988). To prevent the misfolding of protein under stressful events, the unfolded protein response (UPR) is initiated to maintain the correct folding of proteins, and this process manages the proper level of protein influx into the endoplasmic reticulum (ER), which is the main organelle involved in protein processing (Boelens et al., 2007; Cao and Kaufman, 2012; Hampton, 2000; Hetz, 2012). Once there is overload of misfolded proteins in the ER, the ER stress mechanism is active, which is controlled by three effectors on the ER lumen characterized as protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor (ATF6) and inositol-requiring enzyme 1 (IRE1) (Boyce and Yuan, 2006). The activation of the effectors initiates various cellular processes that are related to maintenance of cellular homeostasis. For example, the IRE1 effector molecule controls ER stress through translational suppression by mRNA degradation and activation of stress-activated protein kinase (SAPK) signaling (Chen and Brandizzi, 2013; Hollien et al., 2009). SAPK signaling is a conserved cellular signaling mechanism to conduct information about the internal and external cellular stress, and this process is controlled by the activation of series of mitogen-activated protein kinases (MAPKs). This is known to be initiated by the activation of the TNF receptor associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) that triggers the activity of c-Jun N-terminal kinases (JNK) initiating the activity of

SAPK signaling (Nishitoh et al., 2002; Urano et al., 2000). The activation of SAPK signaling by ER stress causes the initiation of the mitochondrial pathway of apoptosis through the activation of C/EBP homologous protein (CHOP) (Tournier et al., 2000; Wada and Penninger, 2004; Zinszner et al., 1998). Recent studies shown that CHOP is transcriptionally regulated to cause cell cycle arrest or apoptosis (Barone et al., 1994; Gotoh et al., 2002; Maytin et al., 2001). To combine together, the ER stress and SAPK signaling affects the cellular status during stressful events, and both processes act to initiate apoptosis, which marks the initiation of demolition in the cell.

Apoptosis is a unique cellular process that occurs in function to protect an organism by withdrawal of abnormal cells, and it is tightly controlled by multiple cell death related genes such as caspases (Ferri and Kroemer, 2001; Oyadomari et al., 2002; Thornberry and Lazebnik, 1998). Apoptosis is marked by the phenotypical and genetic change in the cell, and both changes are important in the detection of apoptosis (Kerr et al., 1972). The specific cell death (CED) genes were first identified in the nematodes, and these genes represent the caspase family of proteases that are important in the initiation and execution of apoptosis (Ellis and Horvitz, 1986; Wang and Yang, 2016). Caspases are the core machinery that regulates the apoptotic cascade, which can be divided into two major categories: initiator caspases (e.g. caspase-8 and -9) and executioner caspases (e.g. caspase-3 and -7) (Salvesen and Ashkenazi, 2011). The initiator caspases activates the apoptotic stimuli through oligomerization, and these initiator caspases induce the cleavage of executioner caspases to drive the execution phase of the apoptotic cell death, hence once the executioner

caspases are active, the apoptotic progress is directed to cellular death (Dix et al., 2008; Mahrus et al., 2008). However, exceptionally in some cases, an apoptotic cell is able to reverse itself from apoptosis, only when it is in the early apoptotic phase, where the cell membrane integrity is still preserved and the executive caspases are not activated (Tang et al., 2012). The cascade of apoptotic caspases is controlled by two signaling pathways: the intrinsic pathway and extrinsic pathway. The intrinsic pathway is also known as the mitochondrial pathway, and it is regulated by the BCL2 family genes, which governs the expression of caspase-activating factors from the mitochondria (Adams and Cory, 1998). On the other hand, the extrinsic pathway is controlled by the specific death receptors, which could receive extracellular signals through death ligands and transmit the signals to the caspase machinery (Ashkenazi and Dixit, 1998).

Another cellular process that is affected by external stress is the cell cycle, which is one of the most important mechanisms among the biological processes that is controlled by multiple pathways and checkpoint proteins as p53 to assure that the cell is replicating without error (Pietsch et al., 2008). The p53 protein is known as a tumor suppressor that monitors the stress signals and inhibits the tumor-cell growth, while it has many other functions as apoptosis regulation and cell cycle arrest (Stambolic et al., 2001; Wagner et al., 1994). The cell cycle arrest controlled by p53 is mediated by the DNA damage, but also can be induced by various stress agents, arresting the cell cycle at the checkpoints of G_2/M or G_1 phase (Agarwal et al., 1995). The types of stress agents could vary including physical stress as coldness, and it was reported that the cell cycle could sensitively react to

temperature change in response to the cold shock (Michalovitz et al., 1990; Owen-Schaub et al., 1995; Rieder and Cole, 2014). Hypothermia is one of the most prominent causes of stress during embryonic dormancy, and it was shown that the cell cycle is arrested in the G_0/G_1 or G_2/M phase during embryonic dormancy (Ewert, 1991). For example, *Austrofundulus limnaeus* embryos are arrested in the G_0/G_1 phase of the cell cycle during embryonic dormancy, while the *Bombyx mori* embryo is arrested in the G_2/M phase (Nakagaki et al., 1991; Podrabsky and Culpepper, 2012).

In summary, shortly after oviposition the avian embryo is known to be exposed to cold stress, which can possibly activate various cellular and molecular stress signals. However, the exact molecular mechanisms and signaling signatures are not elucidated in the dormant avian embryo. Therefore, it will be valuable to investigate the molecular changes that occur in the dormant avian blastoderm, thus providing information about the stress-resistant nature of the avian blastoderm.

Materials and Methods

Experimental animals and animal care

The care and experimental use of White Leghorn (WL) chickens was approved (SNU-150827-1) by the Institute of Laboratory Animal Resources, Seoul National University, Korea. For fertilized eggs, chickens were maintained according to a standard management program at the University Animal Farm. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

Sample preparation

Eyal-Giladi and Kochav (EGK) stage X (Eyal-Giladi and Kochav, 1976) avian blastoderms were collected from White Leghorn chickens, and kept at in a chamber at 16°C for 6hours or 7days. For incubated samples, eggs stored at 16ºC for 6hours were incubated in a chamber at 37.5°C with 80% humidity for 4 hours. Accordingly the blastodermal cells were harvested and gently washed with PBS at room temperature.

RNA-seq analysis

Total RNA was isolated from each blastoderm group using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's procedure. Quality control of extracted total RNA was measured by Nanodrop 2000 and Bioanlyzer 2100. 1μg of total RNA were purified by poly-A selection and used for the construction of cDNA libraries with the kit TruSeq RNA sample prep (Illumina Inc., San Diego, CA). The resulting libraries were used to generate high-

throughput NGS data using the Illumina HiSeq 2000 (Illumina Inc., San Diego, CA) platform to produce paired 101-bp reads. The raw sequencing outputs were processed for the removal of low quality bases (quality cutoff; Q20), reads shorter than 90-bp and the trimming of Illumina adapters. To mapping the reads on chicken genome, the reads were mapped onto the reference genome from the NCBI (Gallus gallus v.4) by Tophat 2.0.846. Total gene read counts were calculated on FPKM by Cufflinks 2.0.246. Differentially expressed genes of samples were identified by Kal's Z-test (p-value < 0.05, fold change > 2 or < 0.05) on proportions in CLC Genomics Workbench 6.0.4 (CLC Bio, Aarhus, Denmark). Hierarchical clustering with Euclidean distance and single linkage method, principal component analysis and volcano plot were performed with the CLC Genomics Workbench. Gene ontology analysis was performed using DAVID47 (http://david.abcc.ncifcrf.gov/) and selected with p-value less than 0.05. The significantly up-regulated and down-regulated genes in each sample were analyzed for the Kyoto Encyclopedia of Genes and Genomes48 (http://www.genome.jp/kegg/) pathway and for protein network by STRING49.

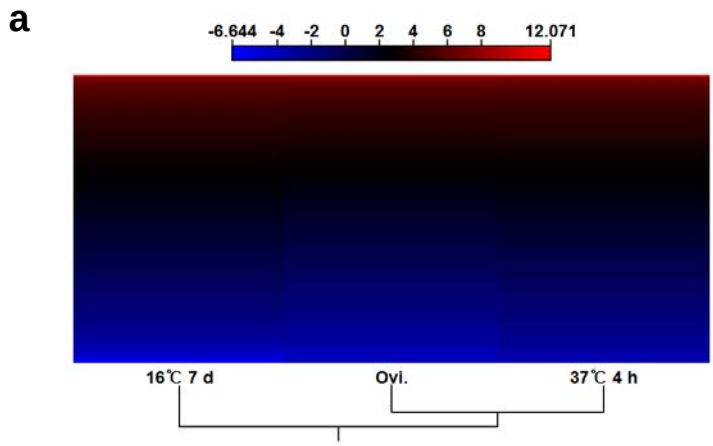
Results

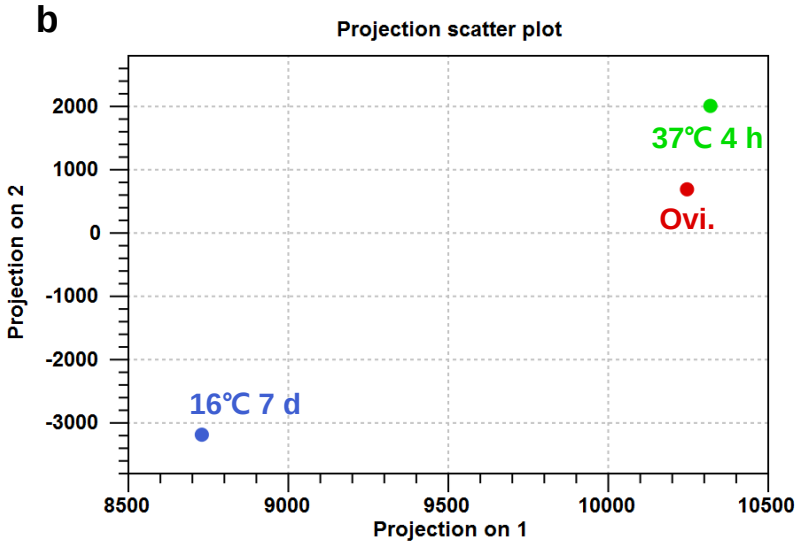
The transcriptome of the chicken embryo was analyzed by RNA sequencing of the immediately ovipositioned, 7 day post-ovipositioned and 37ºC 4 hours incubated Stage X blastoderm cells. The differentially expressed genes (DEGs) were projected on a heat map (Fig 1a), and principal component analysis was conducted among the three groups, which shown that the transcriptome of the 6 hours post-ovipositioned and 37ºC 4 hours incubated had more similarity compared to the 7 days post-ovipositioned blastoderm transcriptome (Fig 1b). Also the fold change of DEGs compared to the 6 hours post-ovipositioned blastoderm was observed (Fig 1c). In the 37ºC 4 hours incubated blastoderm, total 361 genes were up-regulated and 109 genes were down regulated, while in the 7 days post-ovipositioned blastoderm 502 genes were up-regulated and 483 genes were down-regulated (Fig 1c). It was shown that 132 up-regulated genes and 160 down-regulated genes overlapped in the 37ºC 4 hours incubated and 7 days post-ovipositioned blastoderm.

Furthermore, the DEGs of 6 hours post-ovipositioned and 7 days post-ovipositioned blastoderm was compared, and transcripts that were up or down regulated by 2-fold with p-value less than 0.05 were selected (Fig 2a, b). The gene ontology terminology (GO term) was enriched for up and down regulated genes, which has indicated that endoplasmic reticulum (ER) stress and ER related processes were mainly up-regulated after oviposition for 7 days (Fig 2c). KEGG pathway analysis shown that MAP kinase-signaling pathway was mostly up-regulated at 7 days post-oviposition (Fig 3). At the same

time, the ER protein processing related process was mostly up-regulated at 7 days post-ovipsition (Fig 3). Also, the protein-signaling network was analyzed using STRING49, and the results shown similar process as the KEGG pathway analysis was activated in each storage or incubation condition. Especially, HSPA5 and JUN related protein signaling were up-regulated at 7 days post-oviposition, while HSP90AA1 related protein network was down-regulated after 7 days post-oviposition (Fig 4).

The same analysis was performed for the 6 hours post-ovipositioned and 37ºC 4 hours incubated blastoderm transcripts (Fig 5a, b). The GO term enrichment results shown that embryo development and organogenesis related process were up-regulated during 37ºC 4 hours incubation (Fig 5c). Also, the KEGG pathway analysis shown that nucleotide/ribonucleotide biosynthesis and cell cycle related processes were mainly down-regulated at 7 days post-oviposition (Fig 6). Furthermore, the STRING analysis of protein networks shown that protein network related to development is active in the 37ºC 4 hours incubated blastoderm, and especially the HNF4A protein was significant (Fig 7).


a
-6.644 -4 -2 0 2 4 6 8 12.071
16℃ 7 d
Ovi.
37℃ 4 h


b
Projection scatter plot
Projection on 2
2000
1000
0
-1000
-2000
-3000
37℃ 4 h
Ovi.
16℃ 7 d
8500
9000
9500
10000
10500
Projection on 1

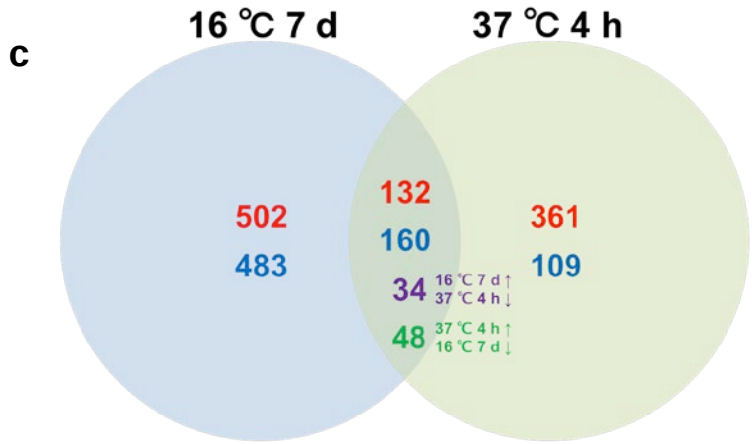
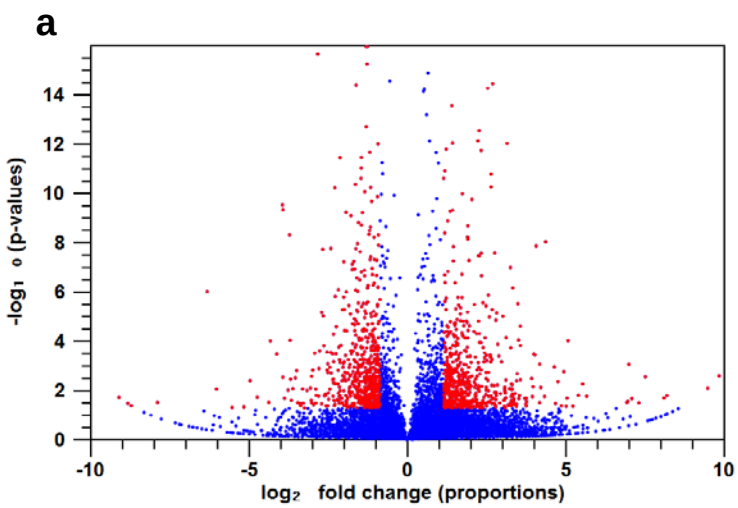
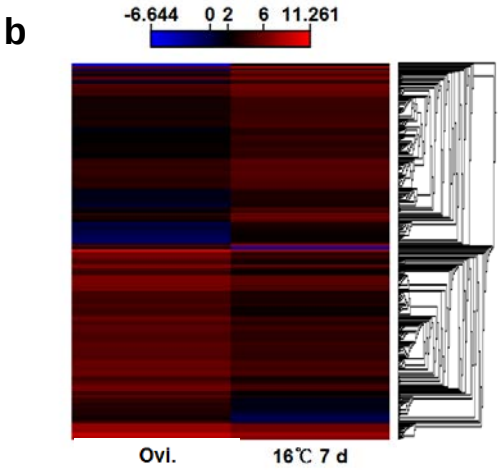
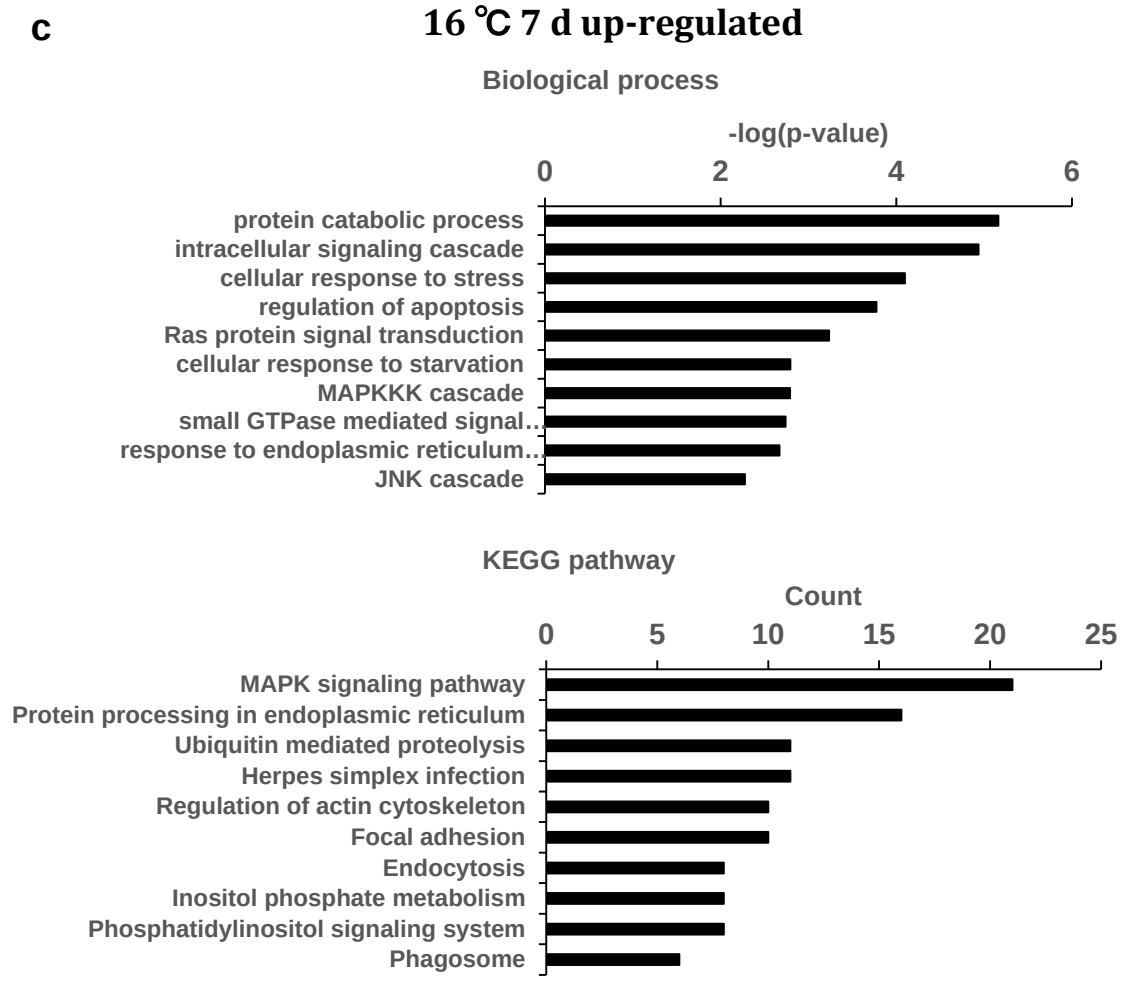

c
16 ℃ 7 d
37 ℃ 4 h
502
483
132
160
34
16 ℃ 7 d↑
37 ℃ 4 h↓
48
37 ℃ 4 h↑
16 ℃ 7 d↓
361
109

Figure 1. Transcriptomic analysis of chicken blastoderm. a, Hierarchical clustering of RNA-seq profiles of chicken blastoderm with Euclidean distance and single linkage method. Each sample was shown as one node. b, Principal component analysis (PCA) plot of the transcriptome of chicken blastoderm with three conditions. Each samples was shown as single color and circle. c, Venn diagram illustration of differentially expressed genes (DEGs) compared to Ovi. blastoderm (Kal's Z-test p-value < 0.05, fold change > 2 or < 0.5). The number of up-regulated and down-regulated genes are shown in red and blue respectively. The number of 16 °C 7 d up-regulated and 37 °C 4 h down-regulated genes are shown in purple, and the number of 37°C 4 h up-regulated and 16°C 7 d up-regulated genes are shown in green. 16 °C 7 d, 7 days stored blastoderm at 16 °C; Ovi, ovipositioned blastoderm; 37 °C 4 h, 4-h incubated blastoderm at 37 °C.


a
14
12
10
8
6
4
2
0
-log₁₀ (p-values)
-10
-5
0
5
10
log₂ fold change (proportions)


b
-6.644
0 2
6
11.261
Ovi.
16℃ 7 d


c
16 ℃ 7 d up-regulated
Biological process
-log(p-value)
0
2
4
6
protein catabolic process
intracellular signaling cascade
cellular response to stress
regulation of apoptosis
Ras protein signal transduction
cellular response to starvation
MAPKKK cascade
small GTPase mediated signal…
response to endoplasmic reticulum…
JNK cascade
KEGG pathway
Count
0
5
10
15
20
25
MAPK signaling pathway
Protein processing in endoplasmic reticulum
Ubiquitin mediated proteolysis
Herpes simplex infection
Regulation of actin cytoskeleton
Focal adhesion
Endocytosis
Inositol phosphate metabolism
Phosphatidylinositol signaling system
Phagosome

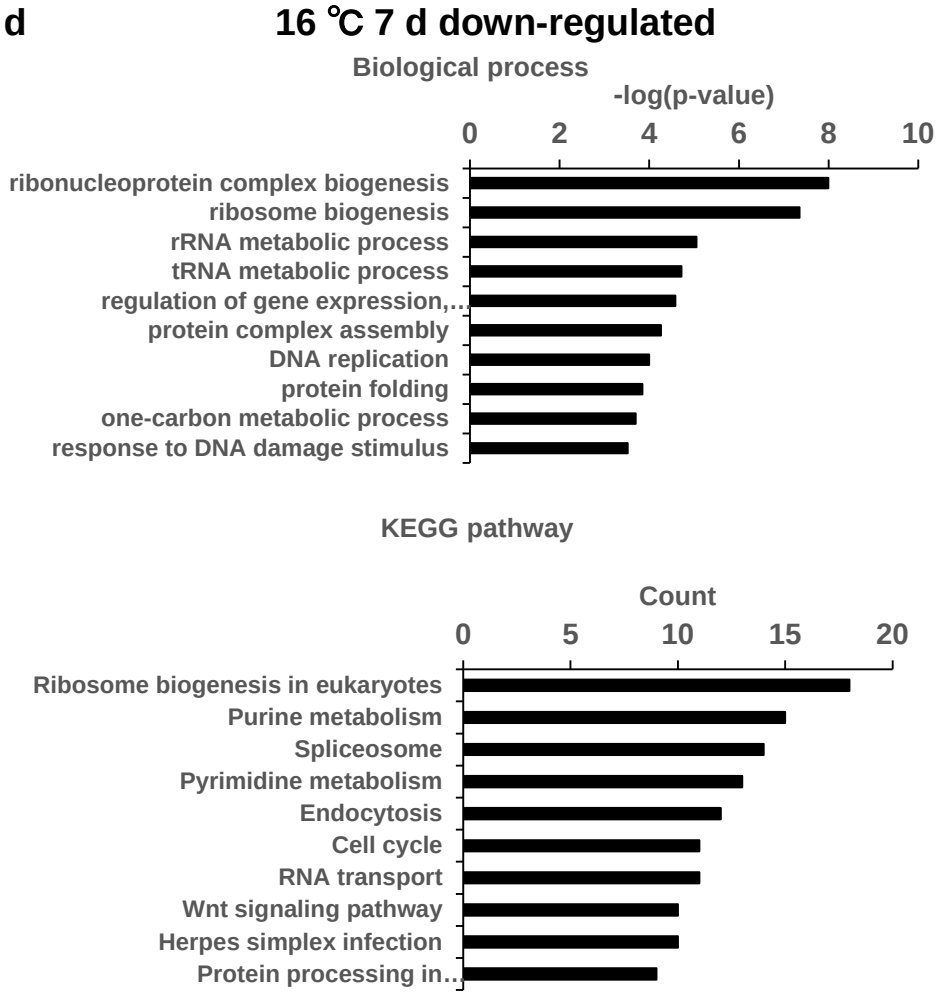
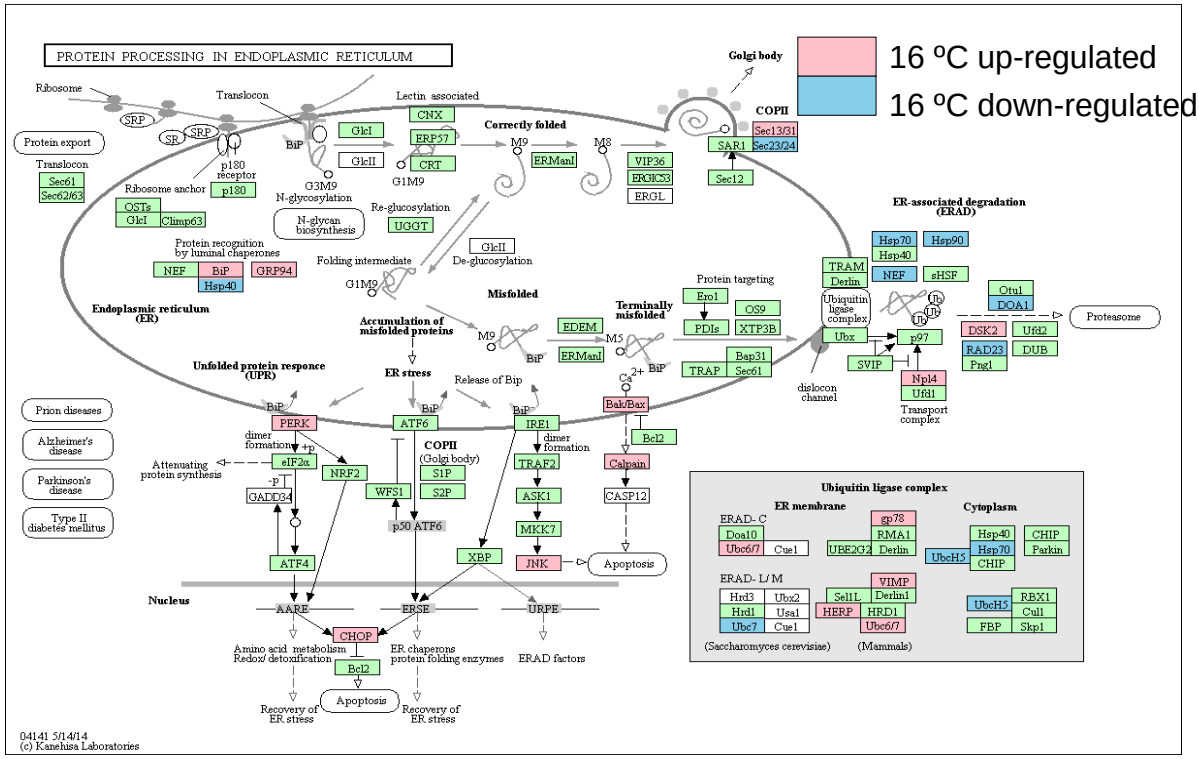


Figure 2. Gene expression and gene set enrichment analysis during chicken blastoderm dormancy. a, Volcano plot of transcriptome analysis between 16°C 7 d and Ovi. blastoderm. Red dots indicate differentially expressed genes in 16°C 7 d blastoderm (Kal's Z-test p-value < 0.05, Fold change > 2 or < 0.5). b, hierarchical clustering of differentially expressed genes of 16°C 7 d blastoderm compared to Ovi. blastoderm with Euclidean distance and single linkage method. Gene ontology (GO) enrichment analysis of biological process and KEGG pathway of up-regulated (c) and down-regulated (d) genes in 16°C 7 d blastoderm. Top 10 enriched terms and pathways were shown. Redundant GO terms were removed.


PROTEIN PROCESSING IN ENDOPLASMIC RETICULUM
16 ºC up-regulated
16 ºC down-regulated
Golgi body
COPII
Ribosome
Translocon
Lectin associated
Protein export
Correctly folded
Endoplasmic reticulum (ER)
Misfolded
Terminally misfolded
Accumulation of misfolded proteins
ER-associated degradation (ERAD)
Proteasome
Unfolded protein responce (UPR)
ER stress
Release of Bip
Folding intermediate
Protein targeting
Protein recognition by luminal chaperones
N-glycosylation
N-glycan biosynthesis
Re-glucosylation
De-glucosylation
Ubiquitin ligase complex
Transport complex
dislocon channel
Prion diseases
Alzheimer's disease
Parkinson's disease
Type II diabetes mellitus
Attenuating protein synthesis
COPII (Golgi body)
Nucleus
Amino acid metabolism Redox/ detoxification
ER chaperons protein folding enzymes
ERAD factors
Recovery of ER stress
Apoptosis
Ubiquitin ligase complex
ER membrane
Cytoplasm
(Saccharomyces cerevisiae)
(Mammals)
04141 5/14/14
(c) Kanehisa Laboratories

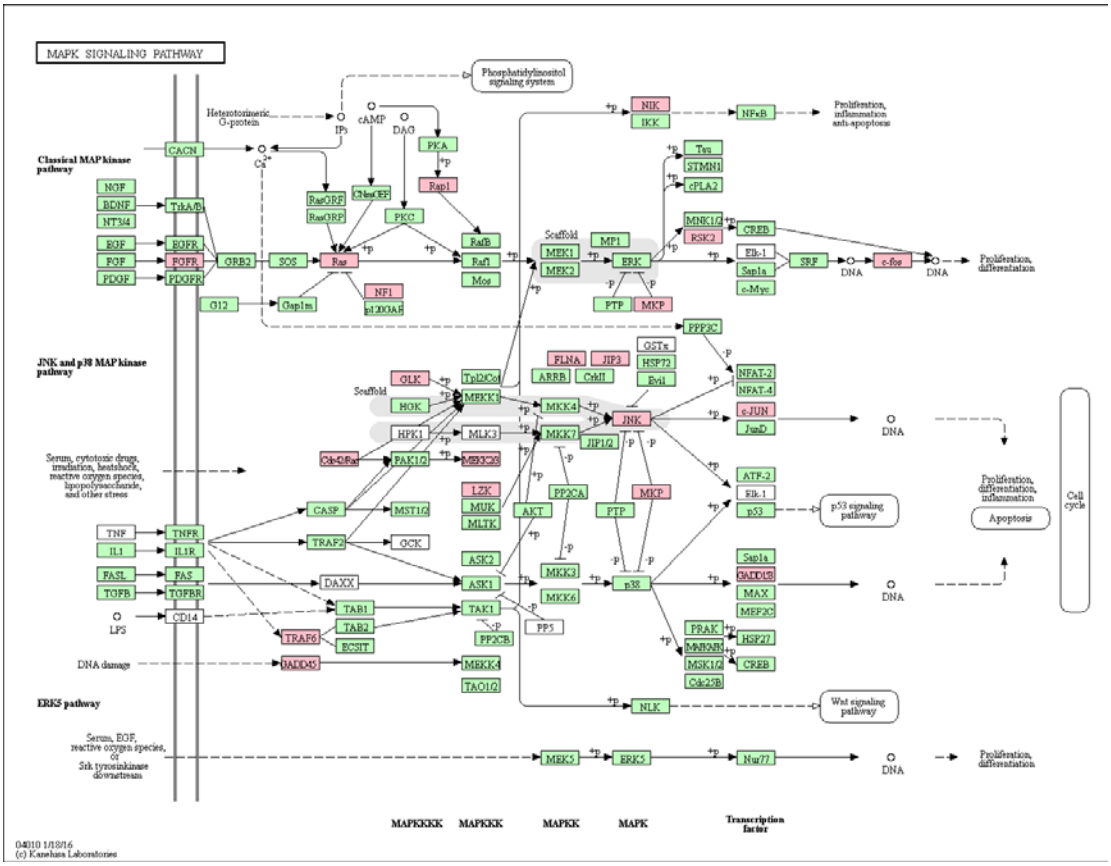
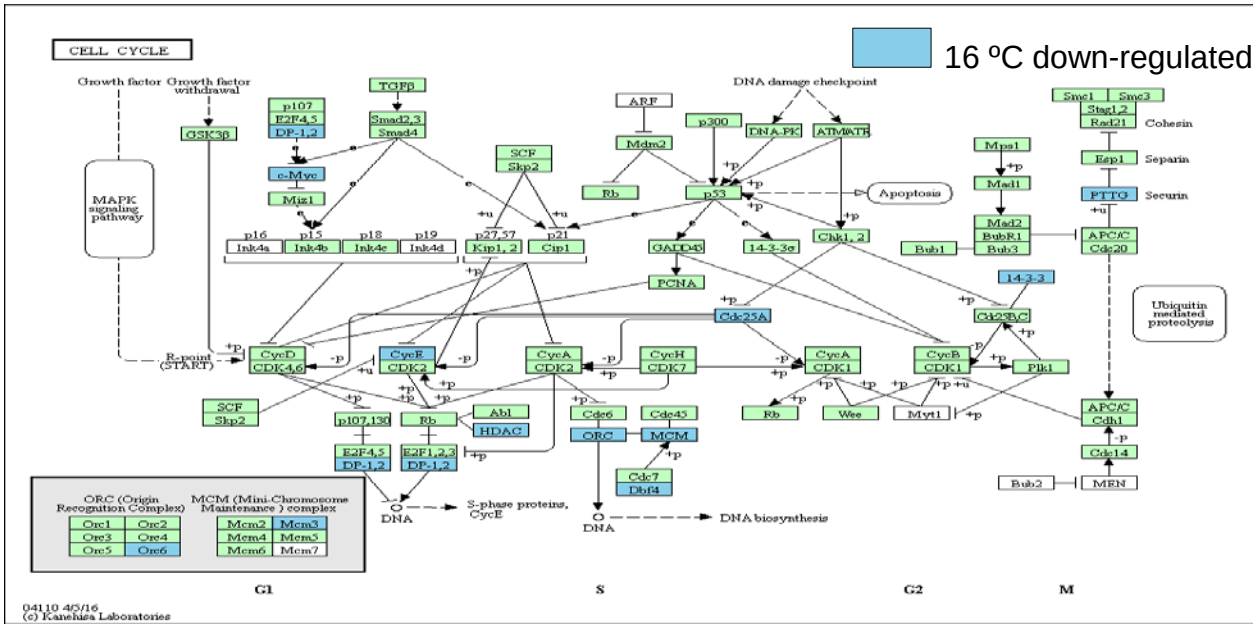
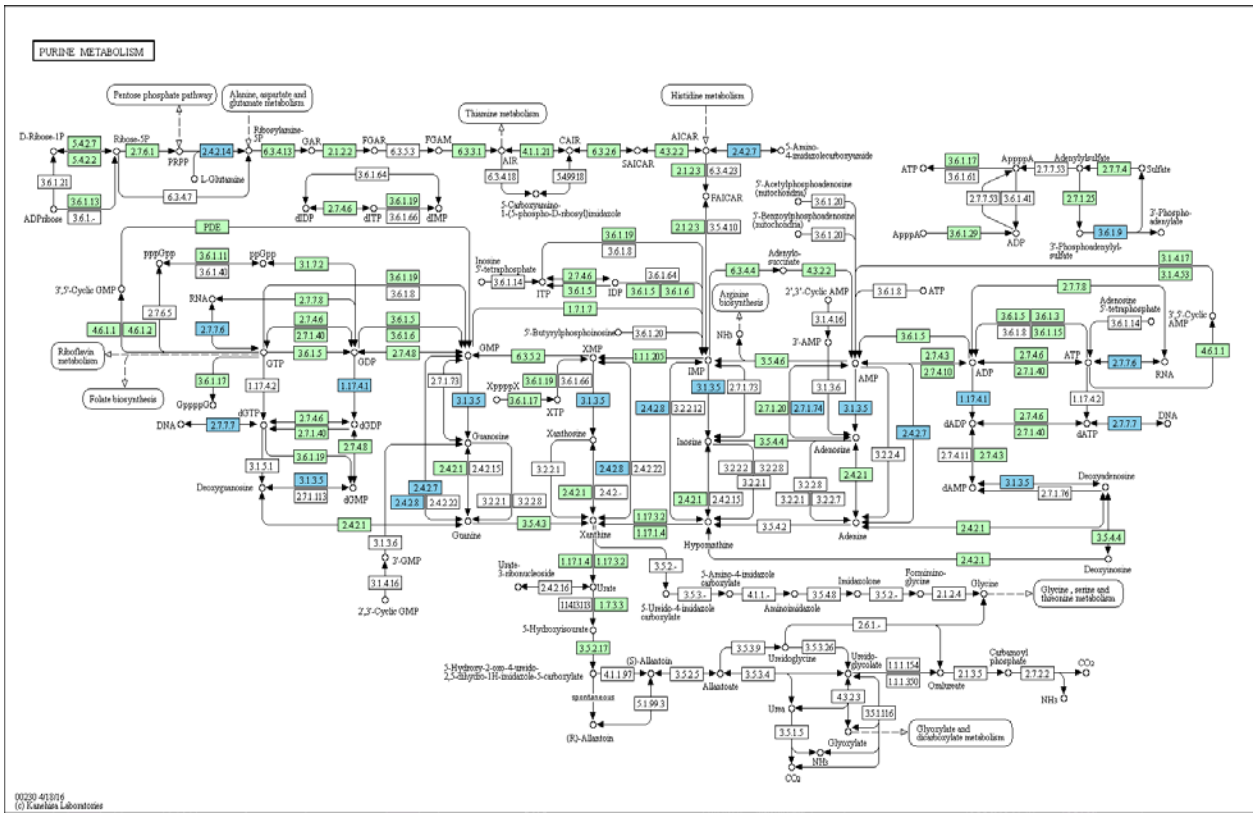

MAPK SIGNALING PATHWAY
Classical MAP kinase pathway
JNK and p38 MAP kinase pathway
ERK5 pathway
Scaffold
Proliferation, differentiation
Cell cycle
Apoptosis
DNA
MAPKKK
MAPKKK
MAPKK
MAPK
Transcription factor
04010 1/18/16
(c) Kanehisa Laboratories

Figure 3. The KEGG protein processing in endoplasmic reticulum and MAPK signaling pathway of up-regulated and down-regulated genes in 16°C 7 d blastoderm. The pink boxes indicate the up-regulated genes and the pale blue boxes indicate the down-regulated genes in 16°C 7 d blastoderm compared to Ovi. blastoderm.


16 ºC down-regulated
CELL CYCLE
Growth factor
Growth factor withdrawal
MAPK signaling pathway
GSK3β
TGFβ
p107
E2F4,5
DP-1,2
Smad2,3
Smad4
c-Myc
Mxi1
SCF
Skp2
ARF
Mdm2
p300
DNA-PK
ATM/ATR
DNA damage checkpoint
Rb
p53
Apoptosis
GADD45
14-3-3σ
Chk1, 2
p16
Ink4a
p15
Ink4b
p18
Ink4c
p19
Ink4d
p27,57
Kip1, 2
p21
Cip1
PCNA
Cdc25A
R-point
(START)
CycD
CDK4,6
CycE
CDK2
CycA
CDK2
CycH
CDK7
CycA
CDK1
CycB
CDK1
Plk1
SCF
Skp2
p107,130
Rb
Abl
HDAC
Cdc6
Cdc45
ORC
MCM
Cdc7
Dbf4
E2F4,5
DP-1,2
E2F1,2,3
DP-1,2
Rb
Wee
Myt1
Mps1
Mad1
Mad2
BubR1
Bub3
Bub1
Bub2
MEN
14-3-3
Cdc25C
Smc1
Smc3
Stag1,2
Rad21
Cohesin
Esp1
Separin
PTTG
Securin
APC/C
Cdc20
APC/C
Cdh1
Cdc14
Ubiquitin mediated proteolysis
ORC (Origin Recognition Complex)
Orc1
Orc2
Orc3
Orc4
Orc5
Orc6
MCM (Mini-Chromosome Maintenance) complex
Mcm2
Mcm3
Mcm4
Mcm5
Mcm6
Mcm7
DNA
S-phase proteins, CycE
DNA
DNA biosynthesis
G1
S
G2
M
04110 4/5/16
(c) Kanehisa Laboratories


PURINE METABOLISM
Pentose phosphate pathway
Alanine, aspartate and glutamate metabolism
Thiamine metabolism
Histidine metabolism
Folate biosynthesis
Riboflavin metabolism
Arginine biosynthesis
Glycine, serine and threonine metabolism
Glyoxylate and dicarboxylate metabolism
00230 4/18/16
(c) Kanehisa Laboratories

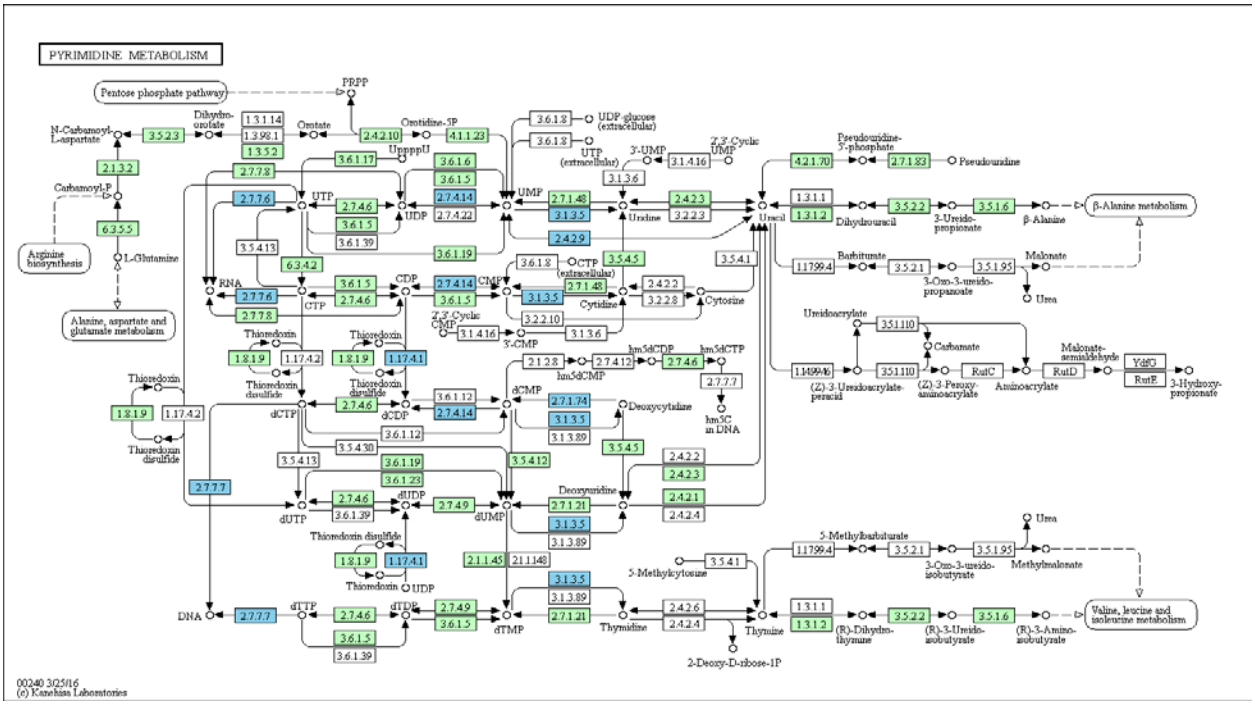


Figure 4. The KEGG cell cycle and nucleotide metabolism including purine and pyrimidine of down-regulated genes in 16°C 7 d blastoderm. The pale blue boxes indicate the down-regulated genes in 16°C 7 d blastoderm compared to Ovi. blastoderm.

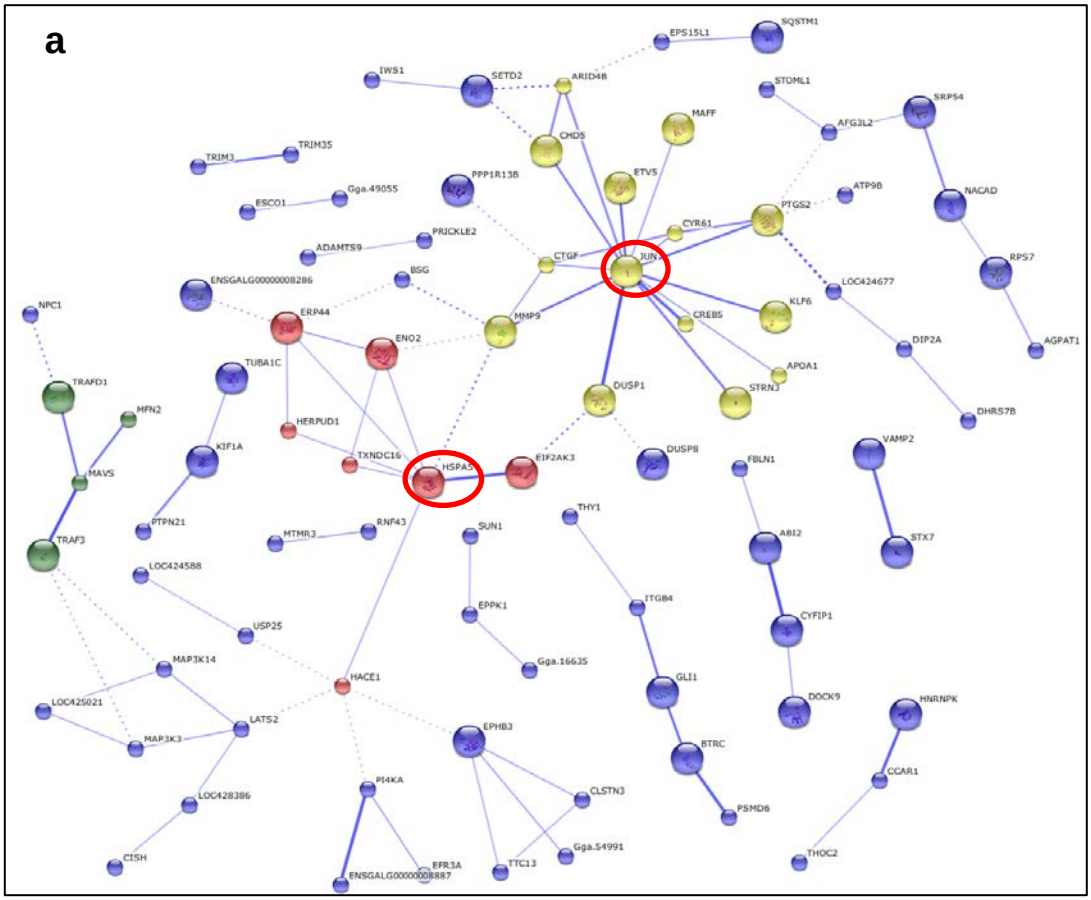
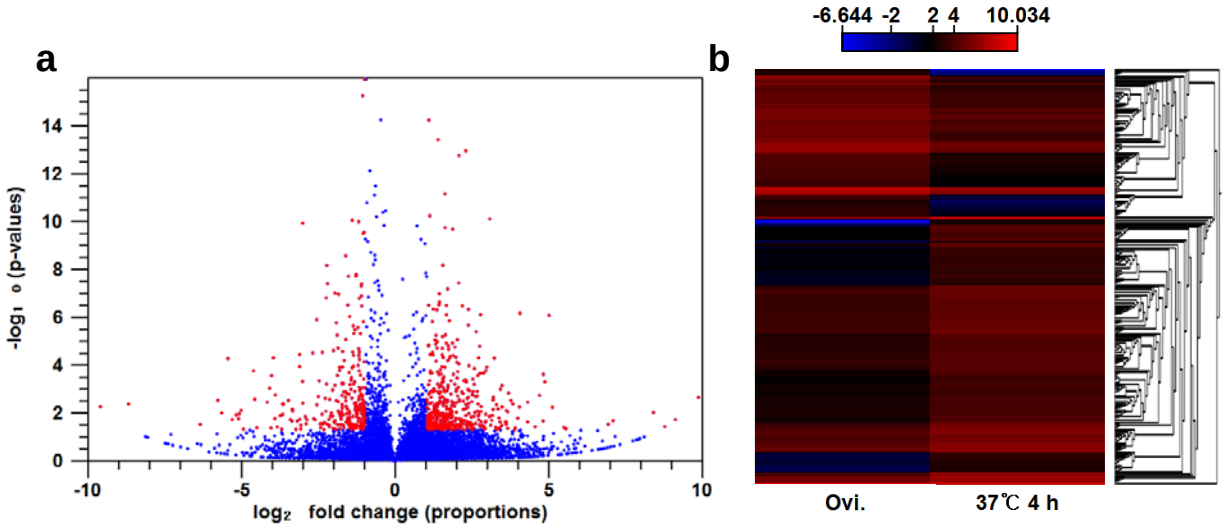
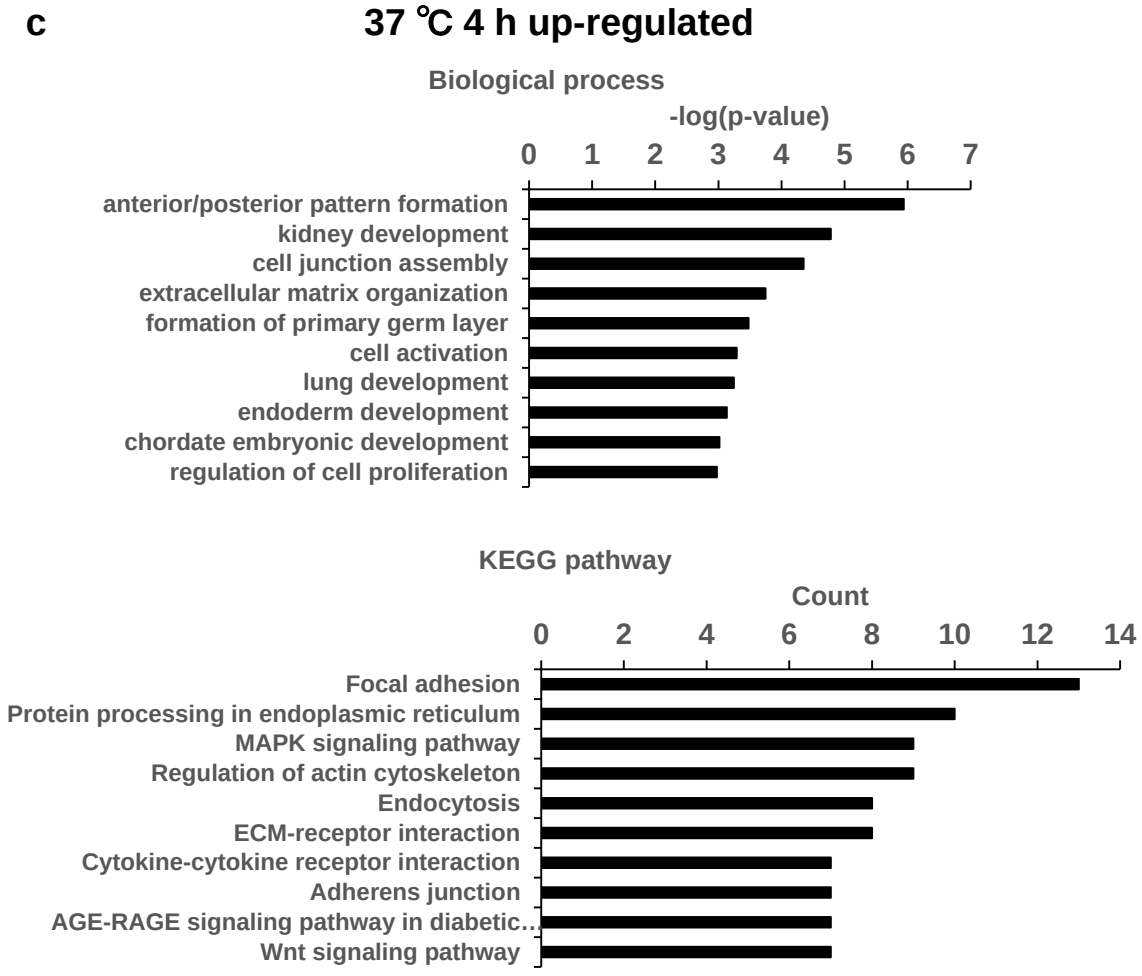

a
EPS15L1
SQSTM1
IWS1
SETD2
ARID4B
STOML1
SRPS4
MAFF
AFG3L2
CHD8
TRIM3
TRIM35
PPP1R13B
ETV5
ATP9B
NACAD
ESCO1
Gga.49055
PTGS2
CYR61
PRICKLE2
ADAMTS9
CTGF
JUN
RPS7
BSG
LOC424677
ENSGALG00000008286
NPC1
ERP44
MMP9
KLF6
CREB5
ENO2
DIP2A
AGPAT1
TUBA1C
APOA1
TRAFD1
DUSP1
STRN3
MFN2
HERPUD1
DHRS7B
KIF1A
TXNDC16
HSPA5
EIF2AK3
DUSP8
FBLN1
VAMP2
MAVS
PTPN21
RNF43
MTMR3
SUN1
THY1
A8I2
STX7
TRAF3
LOC424588
EPPK1
ITGB4
CYFIP1
USP25
Gga.16635
MAP3K14
HACE1
GLI1
DOCK9
HNRNPK
LOC425021
LATS2
MAP3K3
EPHB3
BTRC
CCAR1
PI4KA
CLSTN3
PSMD6
LOC428386
CISH
Gga.54991
THOC2
TTC13
EFR3A
ENSGALG00000008887
b
LARP1
PABPC4
UBA3
ENSGALG00000016189
TFDP1
RRM2
SEMA3A
SERPINH1
NT5C2
ENPP3
RANBP3
LIN28A
PRDX3
MTRF1
ERBB2
KPNA7
HSP90AA1
EYA3
HEMK1
ARL4C
HSPH1
SHMT1
ATF1
AP2B1
ABCG2
LOC417253
KIAA0664
ST14
PPAT
TSKU
EPHA1
RHEB
EZH2
PUS3
ATP13A3
SLC16A10
TSC22D1
SF3B1
CCAR1
LMNB1
APTX
ENSGALG00000015620

Figure 5. Protein network of differentially expressed genes in 16°C 7 d blastoderm. The up-regulated (a) and down-regulated (b) genes in 16°C 7 d blastoderm compared to Ovi. blastoderm (Kal's Z-test p-value < 0.05, Fold change > 4 or < 0.25). Protein network constructed based on STRING database. The hub genes in each network are presented in red circles.


a
b
-6.644 -2 2 4 10.034
-log10 (p-values)
log2 fold change (proportions)
Ovi.
37℃ 4 h


c
37 ℃ 4 h up-regulated
Biological process
-log(p-value)
anterior/posterior pattern formation
kidney development
cell junction assembly
extracellular matrix organization
formation of primary germ layer
cell activation
lung development
endoderm development
chordate embryonic development
regulation of cell proliferation
KEGG pathway
Count
Focal adhesion
Protein processing in endoplasmic reticulum
MAPK signaling pathway
Regulation of actin cytoskeleton
Endocytosis
ECM-receptor interaction
Cytokine-cytokine receptor interaction
Adherens junction
AGE-RAGE signaling pathway in diabetic...
Wnt signaling pathway

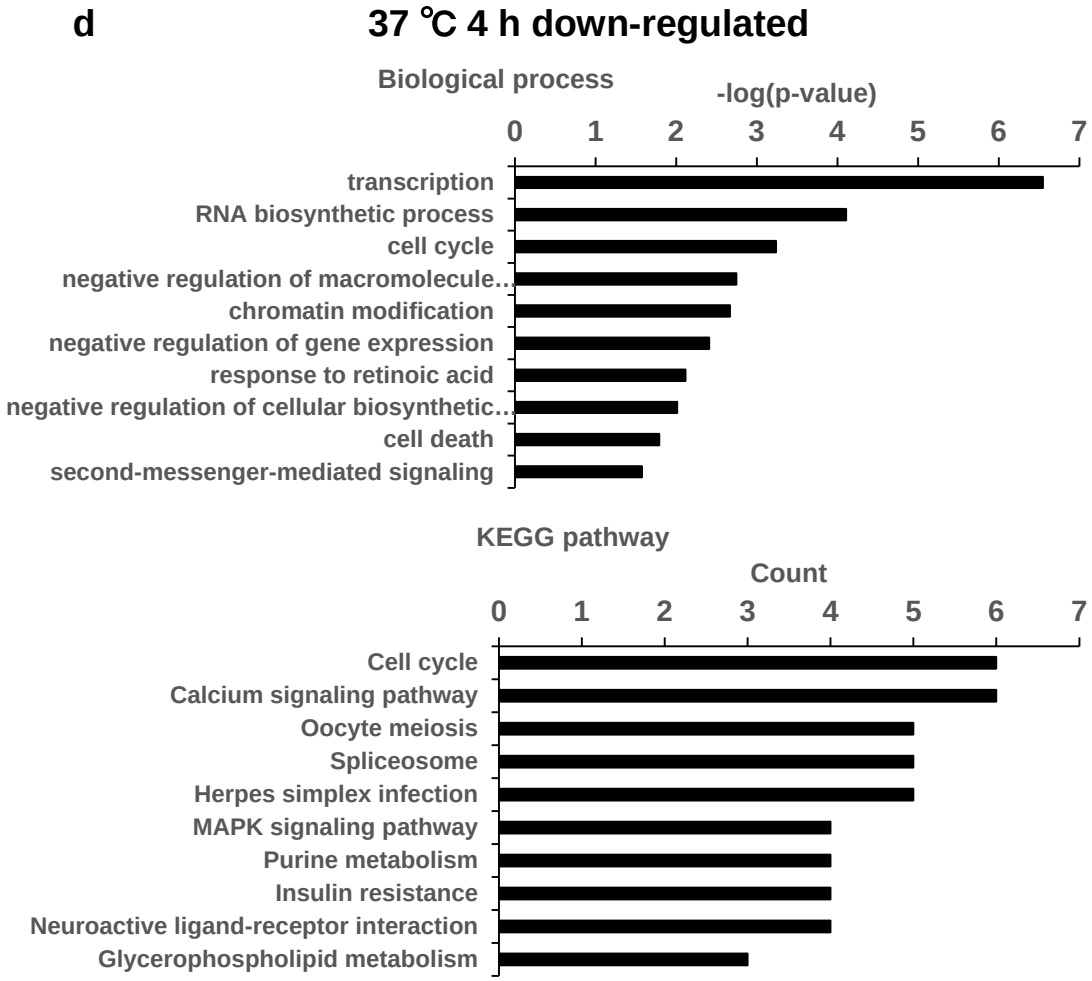


Figure 6. Gene expression and gene set enrichment analysis during initiation of development in chicken blastoderm. a, Volcano plot of transcriptome analysis between 37°C 4 h and Ovi. blastoderm. Red dots indicate differentially expressed genes in 37°C 4 h blastoderm (Kal's Z-test p-value < 0.05, Fold change > 2 or < 0.5). b, hierarchical clustering of differentially expressed genes of 37°C 4 h blastoderm compared to Ovi. blastoderm with Euclidean distance and single linkage method. Gene ontology (GO) enrichment analysis of biological process and KEGG pathway of up-regulated (c) and down-regulated (d) genes in 37°C 4 h blastoderm. Top 10 enriched terms and pathways were shown. Redundant GO terms were removed.

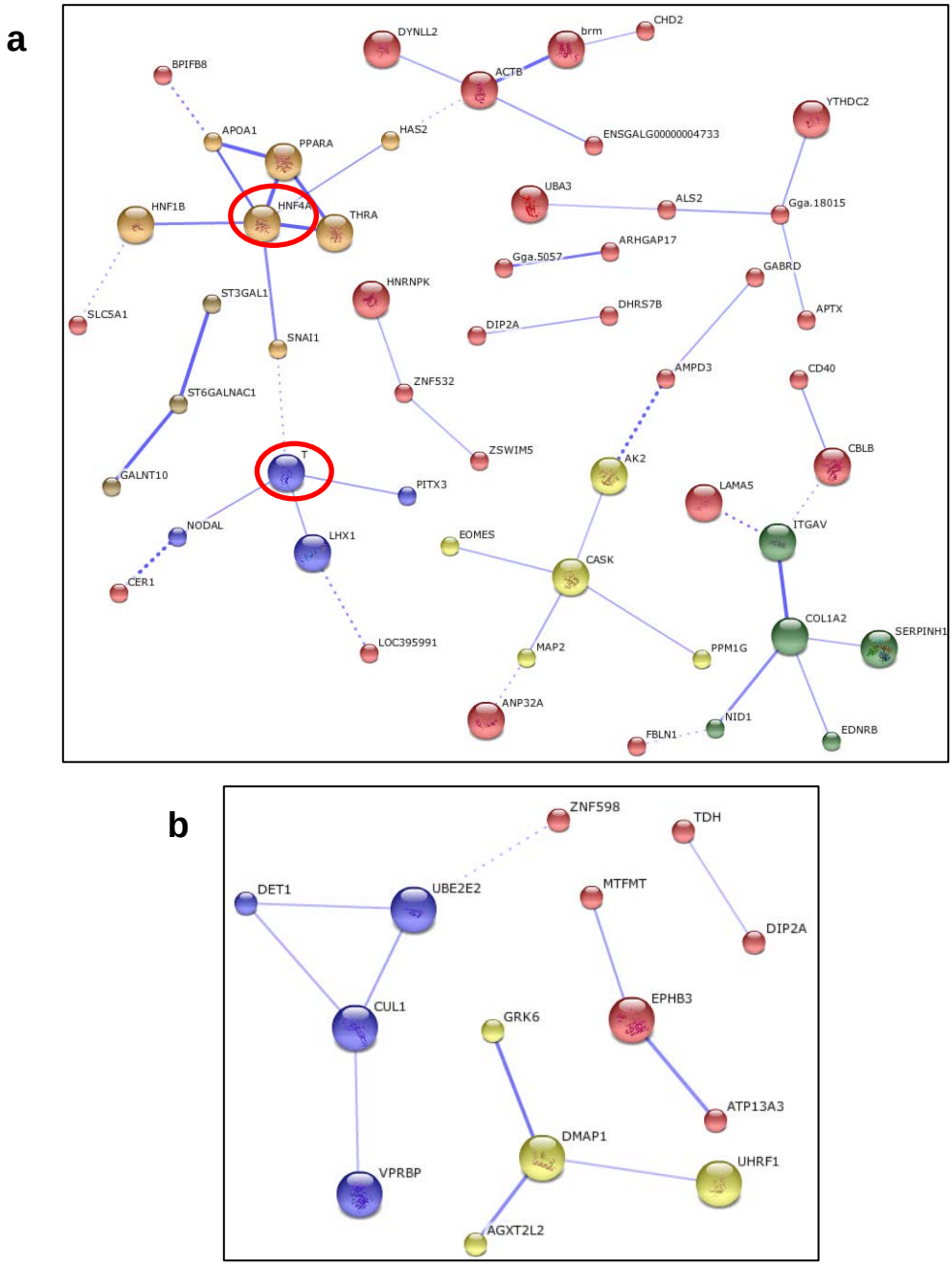


Figure 7. Protein network of differentially expressed genes in 37°C 4 h blastoderm. The up-regulated (a) and down-regulated (b) genes in 37°C 4 h blastoderm compared to Ovi. blastoderm (Kal's Z-test p-value < 0.05, Fold change > 4 or < 0.25). Protein network constructed based on STRING database. The hub genes in each network are presented in red circles.

Discussion

The early avian embryo suspends the development after oviposition due to the low temperature that does not meet the developmental requirements. Although the developmental pause occurs in the early avian embryo, it is able to proceed normal development if adequate temperature is met (Ewert, 1991). However, the molecular processes that are underlying the stress tolerance of the dormant avian embryo are still unknown. Therefore, to investigate the molecular processes those are active in the dormant avian embryo, the transcriptome of the 6 hours and 7 days post-ovipositioned and 37ºC incubated avian embryo were analyzed to observe significant molecular processes that are active.

First of all, the principal component analysis was conducted to identify the transcriptomal similarity between the samples. The results indicated that the 6 hours post-ovipositioned and 37ºC 4 hours incubated embryos were more closely clustered compared to the 7 days post-ovipositioned embryo suggesting that the transcription pattern of genes in the embryos that did not tolerate cold stress were more similar. This could imply that the expression of dormancy or stress related genes largely change the transcription pattern of the avian embryo.

To investigate the processes that are activated during dormancy in more detail, the gene ontology (GO) term enrichment was investigated. In the 16ºC 7 d storaged sample, it was shown that the processes that were related to endoplasmic reticulum (ER) protein processing were highly up-regulated after 7 days of oviposition,

suggesting the possibility of ER stress related process to be active during cold stress of blastoderms. This is supported by the high expression and up-regulation of *HSPA5* and *JUN*, which are related to ER stress and map kinase signaling. The *HSPA5* gene is a HSP70 family gene of which its function is to mainly bind and stabilize unfolded proteins, and activate the ER stress cascade by binding to the ER stress effectors (Mayer, 2013).

Another molecular process that was highly up-regulated based on the GO term enrichment was the MAPK signaling, indicating the strong activation of cellular signaling during the storage of avian embryo. MAPK signaling consists of various types of kinases that are related to cellular processes, and especially the MAPK signaling that is related to recognition of stress is known as the stress-activated protein kinase (SAPK) signaling. SAPK signaling consists of three map kinases as ERK, JNK and p38 (Chang and Karin, 2001). Each map kinase has a unique mechanism in signaling cellular stress response, and usually the final cellular process is related to cell death. The KEGG pathway analysis results show that the MAPK signaling related genes were up-regulated after storage at 16ºC for 7 days. Especially, the *JNK* gene was highly up-regulated indicating the activation of the SAPK signaling pathway. On the other hand, the *JUN* gene is a downstream transcription factor of the stress-activated protein kinase (SAPK) signaling, and it is known to interact with HSP70 family chaperones to induce cell death (Gabai et al., 2000). Collectively, the differentially expressed genes (DEGs) and its clustering suggest that ER stress and SAPK signaling is active in the avian blastoderm, with the support of KEGG pathway analysis that shows the upregulation of

ER stress and SAPK signaling related genes. Especially the SAPK downstream transcription factors *JUN* and *GADD153* were up-regulated after 7 days of storage.

Meanwhile, the processes that are related to purine/pyrimidine metabolism or cell cycle regulation were significantly down-regulated during storage at 16ºC for 7 days. This indicates that the nucleotide metabolic activity or cell cycle regulatory genes are inactive. Similarly, there are reports of cell cycle arrest in *Bombyx mori*, while 98% of total cells are arrested during embryonic dormancy (Nakagaki et al., 1991). However, the factor that induces the cell cycle arrest during dormancy is unknown. One of the factors that could cause cell cycle arrest is cyclins and cyclin dependent kinases. Therefore it could be assumed that the cyclin activity could be restricted in the dormant chicken embryo, though deeper investigation should be conducted.

In contrast to the processes that were up-regulated during storage at 16ºC, the cellular process that are active during the initiation of development was analyzed for comparison. Ovipositioned chicken blastoderms were incubated at 37ºC for 4 hours to initiate development, and RNA sequencing analysis was conducted. The DEG in the 37ºC-incubated embryo compared to the ovipositioned embryo shown that genes related to the anterior-posterior pattern formation or development was up-regulated during 4 hours incubation at 37ºC.

Collectively, the transcriptome of the 16ºC 7 days storaged (dormant) embryo compared with the ovipositioned or 37ºC 4 hours incubated embryo is significantly different. During dormancy, genes related to ER stress or MAPK signaling is significantly up-regulated while nucleotide metabolism or cell cycle regulatory genes are down-

regulated. For further investigation, we should conduct experiments to verify expression of those genes, along with the verification of the activity of MAPK signaling or cell cycle regulation. This could improve the current understanding on the molecular events occurring in the dormant chicken embryo.

CHAPTER 4

Molecular and Signaling Signatures in the Dormant Avian Embryo

Introduction

Exposure to acute cold stress is common in most avian species, because of its oviparous nature (Patterson et al., 2008). The ambient temperature of where the egg is laid will vary, but has large difference from the oviductal temperature. The ability of an animal to endure temperature decline is called “cold torpor”, and this phenomenon occurs mostly in hibernating animals or reptilian and avian embryos (Ewert, 1991). The characteristic of cold torpor is to suspend the development during the stressed period, and defend the embryo from damage that could be caused by low temperature. In many avian species, brooding of eggs occur only after the last egg in the clutch has laid (Winkler and Walters, 1983). Therefore, the first egg laid in the clutch will experience a period of moderate to harsh cold torpor, compared to the last egg that is immediately brooded. For example, the domesticated fowl (*Gallus gallus*) eggs are industrially stored at 10°C to 16°C up to 14 days without significantly affecting the hatchability of the chicks (Fasenko, 2007). There were several studies that investigated the internal changes in the eggs during storage, such as pH elevation in the albumen or egg mass decline, but yet there is handful information on the molecular changes in the avian embryo during dormancy (Lapao et al., 1999; Rocha et al., 2013; Walsh et al., 1995). As demonstrated, the avian blastoderm is able to tolerate stress during embryonic dormancy, but the underlying molecular changes or mechanisms are yet investigated. Therefore, studying the molecular changes occurring in the dormant avian embryo will be valuable to

understand the unknown mechanisms that maintain the embryo's homeostasis during dormancy.

At the cellular level, the most immediate change when external stress (temperature, oxygen availability) is present is the protein misfolding. To prevent the misfolding of protein under stressful events, the unfolded protein response (UPR) is initiated to maintain the correct folding of proteins, and this process manages the correct level of protein influx in the endoplasmic reticulum (ER) (Cao and Kaufman, 2012). Once there is overload of misfolded proteins in the ER, the ER stress mechanism is active, which is controlled by three effectors on the ER lumen characterized as PERK, IRE-1 and ATF6 (Sano and Reed, 2013). ER stress is also closely related to stress-activated protein kinase (SAPK) signaling, which is a conserved cellular signaling mechanism to conduct information about the internal and external cellular stress (Cowan, 2003). SAPK signaling can be activated by the ER stress effector IRE-1, which phosphorylates the MAP3K and initiates the signaling (Chen and Brandizzi, 2013). To combine together, the ER stress and SAPK signaling affects the cellular status during stressful events, and both processes act to initiate apoptosis. The initiation of apoptosis marks the initiation of demolition in the cell.

Apoptosis is a unique cellular process, because it is a tightly controlled mechanism of multiple cell death related genes such as caspases. Apoptosis is marked by the phenotypical and genetic change in the cell, and both change are important in the detection of apoptosis. Once the apoptotic process is active, the cell is most likely to undergo programmed cell death. However, in some cases, an

apoptotic cell is able to reverse itself from apoptosis, only when it is in the early apoptotic phase, where the cell membrane integrity is still preserved and the executive caspases are not activated (Tang et al., 2012).

In addition to the activation of apoptotic genes, cell cycle is also affected by exposure to stress. The cell cycle is one of the most important mechanisms among the biological processes, and is controlled by multiple pathways and checkpoint proteins as p53 to assure that the cell is replicating without error. Under stressful conditions, it is known that the cell cycle is arrested at specific points, and especially during cold stress, it is known that the cell cycle is arrested in various types of cultured cells (Rieder and Cole, 2014). Moreover, it was shown that cellular stress could induce mitotic arrest at the G_2 phase, which consequently activates the SAPK signaling thus increasing the cell death signals (Wang et al., 2000).

In summary, shortly after oviposition the avian embryo is known to be exposed to cold stress, which can possibly activate various cellular and molecular stress signals. However, the exact molecular mechanisms and signaling signatures are not elucidated in the dormant avian embryo. Therefore, it will be valuable to investigate the molecular changes that occur in the dormant avian blastoderm, thus providing information about the stress-resistant nature of the avian blastoderm.

Materials and Methods

Experimental animals and animal care

The care and experimental use of White Leghorn (WL) chickens was approved (SNU-150827-1) by the Institute of Laboratory Animal Resources, Seoul National University, Korea. For fertilized eggs, chickens were maintained according to a standard management program at the University Animal Farm. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

Sample preparation

Eyal-Giladi and Kochav (EGK) stage X (Eyal-Giladi and Kochav, 1976) avian blastoderms were collected from White Leghorn chickens, and kept at in a chamber at 16°C. The eggs were kept in the chamber for various time points from 1hour to 14 days or immediately taken for isolation of embryos. For incubated samples, 7 day storaged or immediately ovipositioned eggs were incubated in a chamber at 37.5°C with 80% humidity for 4 hours. Accordingly the blastodermal cells were harvested and gently washed with PBS at room temperature.

Internal egg temperature measurement

White leghorn eggs were collected and stored in the 16°C chamber or immediately taken for measurement of internal temperature. The internal egg temperature was measured using an analog thermometer, and three eggs were used in each time points of storage.

Quantitative real time PCR analysis

Total RNA was isolated using total 5 embryos dissociated in TRIzol reagent (Thermo, Waltham, MA, USA) according to the manufacturer's instructions. For RT-PCR and quantitative real-time PCR analysis of mRNAs, total RNA (1μg) was used as template for cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The cDNA was serially diluted five folds and was quantitatively equalized for PCR amplification. Primers for real-time PCR of each gene transcript were designed using the primer-BLAST program (Table 1; http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The real-time PCR analysis was performed using CFX96 real-time PCR detection system with a C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The qRT-PCR cycling methods were 95 °C for 3 min followed by 40 cycles at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s; melting curve profiles were analyzed for amplicons. Each test sample was run in triplicate. The relative quantification of gene expression analyzed by the 2-ΔΔCt method(Livak and Schmittgen, 2001).

Western blot

Crude protein was isolated from 5 blastodermal cells by dissociation in RIPA lysis buffer (Thermo, Waltham, MA, USA) with protease inhibitor (Sigma, St. Louis, MO, USA) and phosphatase inhibitor (Sigma, St. Louis, MO, USA). Approximately 5ug of protein from total cell lysate was used in each lane for separation in a 10% SDS-PAGE gel. The protein was transferred onto a Hybond 0.45

PVDF membrane (GE Healthcare Bio-sciences, Little Chalfont, UK), and blocked with 3% skim milk for one hour at room temperature (Sigma, St. Louis, MO, USA). Subsequently, the blocked membrane was incubated overnight at 4°C for primary antibody attachment with 1:1,000 dilution. Horseradish peroxidase conjugated secondary antibody (Thermo, Waltham, MA, USA) was attached with 1:4,000 dilutions at room temperature for one hour. The primary antibodies used were as the following: anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-phospho-p38 (Cell Signaling Technology, Danvers, MA, USA), anti-IRE1 (Abcam, Cambridge, UK), anti-Histone H3.1 (Phospho-Ser10) (Signalway, College Park, Maryland) and anti- β-actin (Santa Cruz, Santa Cruz, CA, USA). The expression was visualized using ECL western blotting detection system (GE Healthcare Bio-sciences, Little Chalfont, UK).

Flow cytometry

In purpose to observe the apoptotic status in the blastoderm, Stage X blastoderm cells were washed with phosphate buffered saline, and the AnnexinV-FITC apoptosis detection kit I (BD Biosciences, San Jose, CA, USA) was used as the staining procedure adhered the manufacturer's procedure. Annexin V and PI double-negative cells were considered viable, Annexin V-positive and PI-negative (early apoptotic) cells or Annexin V- and PI-positive (late apoptotic) cells were considered apoptotic and PI only-positive cells were considered necrotic. The stained cells were analyzed using flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, USA). Also, to examine the cell cycle status, the blastoderm cells were stained with propidium

iodide (PI). Blastoderm cells were collected and fixated with 70% ethanol at 4ºC, and the cells were consequently incubated at 37ºC with 1ug RNaseA for 20 minutes. Total 1ug of PI solution (BD Biosciences, San Jose, CA, USA) was used for staining of blastoderm cells at 4ºC for 30 minutes. The stained cells were analyzed using flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, USA).

TUNEL assay

DNA breakage in the blastoderm was detected by TUNEL assay. Stage X blastoderm cells were fixated with 2% paraformaldehyde at room temperature for 15 minutes, and consequently permeabilized with 0.1% Triton X-100 solution at room temperature for 10 minutes. Permeabilized cells were used for DNA breakage labeling with In situ cell death detection kit, TMR red (Roche, Basel, Switzerland). The staining procedure from the manufacturer was followed, and the cells were mounted with DAPI (Vector laboratories, Burlingame, CA, USA). Accordingly the cells were observed under confocal fluorescence microscope (LSM700, Zeiss, Oberkochen, Germany).

Statistical analysis

Significant differences between groups were examined for statistical analysis using student t-test and one-way ANOVA test, compared to Ovi.. P value less than 0.05 indicated statistical significance (****p value < 0.0001, ***p value < 0.001, **p value < 0.01 and *p value < 0.05).

Results

Egg internal temperature change after oviposition

The egg internal temperature was measured after oviposition to observe the approximate point of the initiation of cold stress. Immediately after oviposition, the egg internal temperature was measured in a 20minute interval until it reaches the ambient temperature. The results indicated that the egg internal temperature reached the ambient temperature, which were 16°C within approximately 180 minutes (Figure 1).

Endoplasmic reticulum (ER) stress activation in the avian blastoderm

To observe the extent of endoplasmic reticulum (ER) stress, in the dormant avian embryo, the gene expression pattern was examined using qRT-PCR. Ovipositioned White Leghorn eggs were immediately taken or stored at 16°C for 1 hour, 2 hours, 6 hours, 1 day, 4 days and 7 days or incubated at 37°C for 4 hours for RNA isolation and examination of gene expression. The results indicated that the ER stress related gene expression was up-regulated after 4 days or 7 days of oviposition compared to the ovipositioned status. *HSPA5*, which is also known as the binding immunoglobulin protein (BiP) was shown to be up-regulated by 7 fold after 7 days of storage at 16°C with a p-value of 0.0095. *ATF6* gene was up-regulated by 3.28 fold after 7 days of storage with p-value of 0.0014. The *MBTPS2* gene, which is also known as S2P, was significantly up-regulated at 1 day and 7 days of storage with p-value of 0.01 and 0.0045 respectively. *CHOP* was

gradually up-regulated by after 4 days storage, and was significantly up-regulated by 11.05 fold with a p-value of 0.017. Interestingly, all of the ER stress related gene expression was down-regulated after incubation at 37°C for 4 hours, and the expression level was similar to the ovipositioned status (Figure 2).

Stress activated protein kinase (SAPK) signaling in the avian blastoderm

The gene expression of downstream transcription factors of SAPK signaling was measured by qRT-PCR. Total RNA was isolated from identical samples as the previous result. The qRT-PCR result indicated that *cJUN* was gradually upregulated by 6.13 fold or 9.24 fold during storage at 16°C for 4 days or 7 days respectively compared to the immediately ovipositioned embryo. Similarly the *DUSP8* gene was significantly up-regulated after 4 days of storage and the expression was maintained until 7 days of storage by 3 folds compared to the ovipositioned embryo. In case of the *BCL2* gene, the expression was controlled until 7 days of storage, which was 5.94 fold higher compared to the ovipositioned embryo. Similar to the ER stress related genes, the SAPK related downstream transcription factors were down-regulated after incubation at 37°C for 4 hours, and the expression level was similar to the ovipositioned status (Figure 3a). In addition to the gene expression pattern, the extent of map kinase protein activation was measured by western blot of JNK, phosphorylated JNK and phosphorylated p38. In the chicken Stage X embryo, the level of JNK expression was uniform among storage or incubation periods.

Meanwhile, the phosphorylated JNK and p38 level increased after 1 day of storage at 16°C and the expression level increased at 7 days of storage at 16°C. However, after incubation at 37°C, the phosphorylated JNK and p38 expression decreased as much as the ovipositioned status with or without storage at 16°C (Figure 3b). Similarly in the duck embryo, the expression pattern of JNK and p38 was identical to that of chicken (Figure 3c).

Apoptotic status during avian blastoderm dormancy

To examine the apoptotic status in the avian blastoderm during storage, the chicken blastoderm cells were collected after oviposition, and stained with annexinV-PI for flow cytometry analysis. The results indicated that the amount of live cells (annexinV^-/PI^-) and necrotic cells (annexinV^-/PI^+) gradually decreased after 4 days of storage, which was 47.75% and 32.65% of total cells respectively. The amount of live and necrotic cells continuously decreased until 14 days of storage, which had 28.7% of live cells and 7.82% necrotic cells. On the other hand, the amount of early apoptotic cells (annexinV^+/PI^-) increased after 4 days of storage, with 19.2% of total cells. The amount of early apoptotic cells greatly increased after 7 days and 14 days of storage, which was 57.15% and 61.95% respectively. However, the amount of late apoptotic cells (annexinV^+/PI^+) did not change among the duration of storage with a proportion of less than 1% of total cells. In contrast to the storaged embryos, the blastoderm cells that were incubated at 37°C for 4 hours had similar proportion of cells to the ovipositioned status (Figure 4a,b). At the same time, TUNEL assay was conducted

to measure the amount of DNA double strand breakage in the avian blastoderm cells during storage. The results indicated that there was minor DNA breakage during storage for both 7 days and 14 days.

Cell cycle status in the avian blastoderm during dormancy

The cell cycle status was observed using propidium iodide (PI) staining of blastoderm cells. The results indicated that from oviposition to 1 day of storage, the proportion of blastoderm cells in each phase was even. However, after 2 days of storage, the amount of G_2/M phase arrested cells increased to 50%, and the amount of G_2/M phase arrested cells constantly increased until 14 days of storage of which the amount of arrested cells reached 68%. At the same time, the amount of G_0/G_1 phase and S phase cells decreased to 15% and 16% respectively in the 14 days storaged embryo. However, once the blastoderm cells were incubated at 37ºC, the cell cycle status was reconstituted back to the ovipositioned status (Figure 5a,b). Also the phosphorylated histone H3 marker, which marks the G_2/M transition, increased after storage at 16ºC for 7 days, but decreased after 4 hours of incubation at 37ºC (Figure 5c).

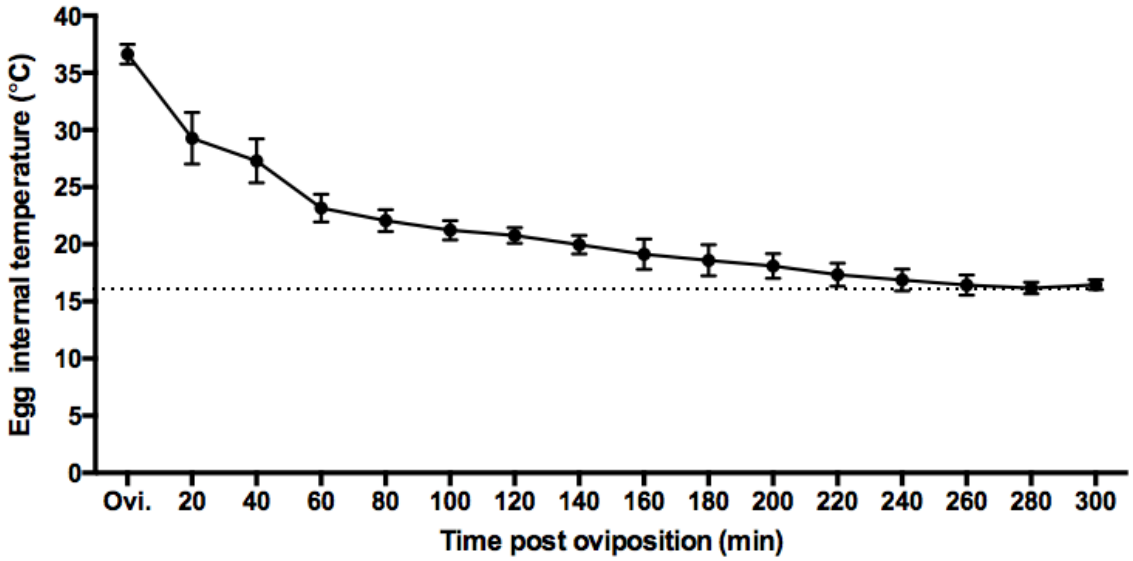


Figure 1. Internal egg temperature change in the storaged avian blastoderms. Ovipositioned eggs were immediately taken or stored at 16ºC for certain amount of time to observe internal egg temperature change. Dotted line indicates ambient temperature (16ºC).

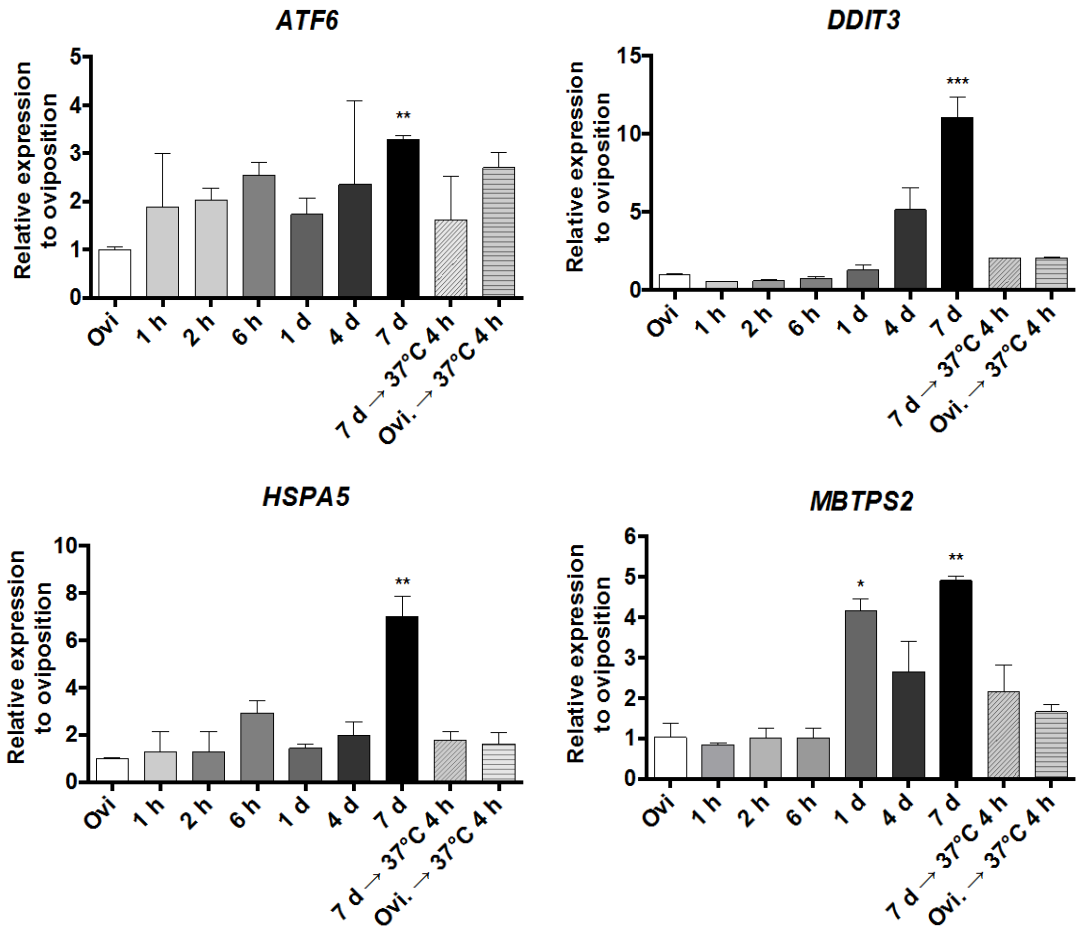


Figure 2. ER stress is activated during chicken embryonic dormancy. Ovipositioned eggs were immediately taken or stored at 16°C for 1 hour, 2 hours, 6 hours, 1 day, 4 days, 7 days or incubated at 37°C for 4 hours for RNA isolation and observation of gene expression. qRT-PCR analysis of ER stress related gene expression was conducted. For statistical analysis, the Student's t-test was used and values were compared to oviposition. ***p value < 0.001, **p value < 0.01, *p value < 0.05. Ovi., immediately ovipositioned embryo; h, hours of storage or incubation; d, days of storage.

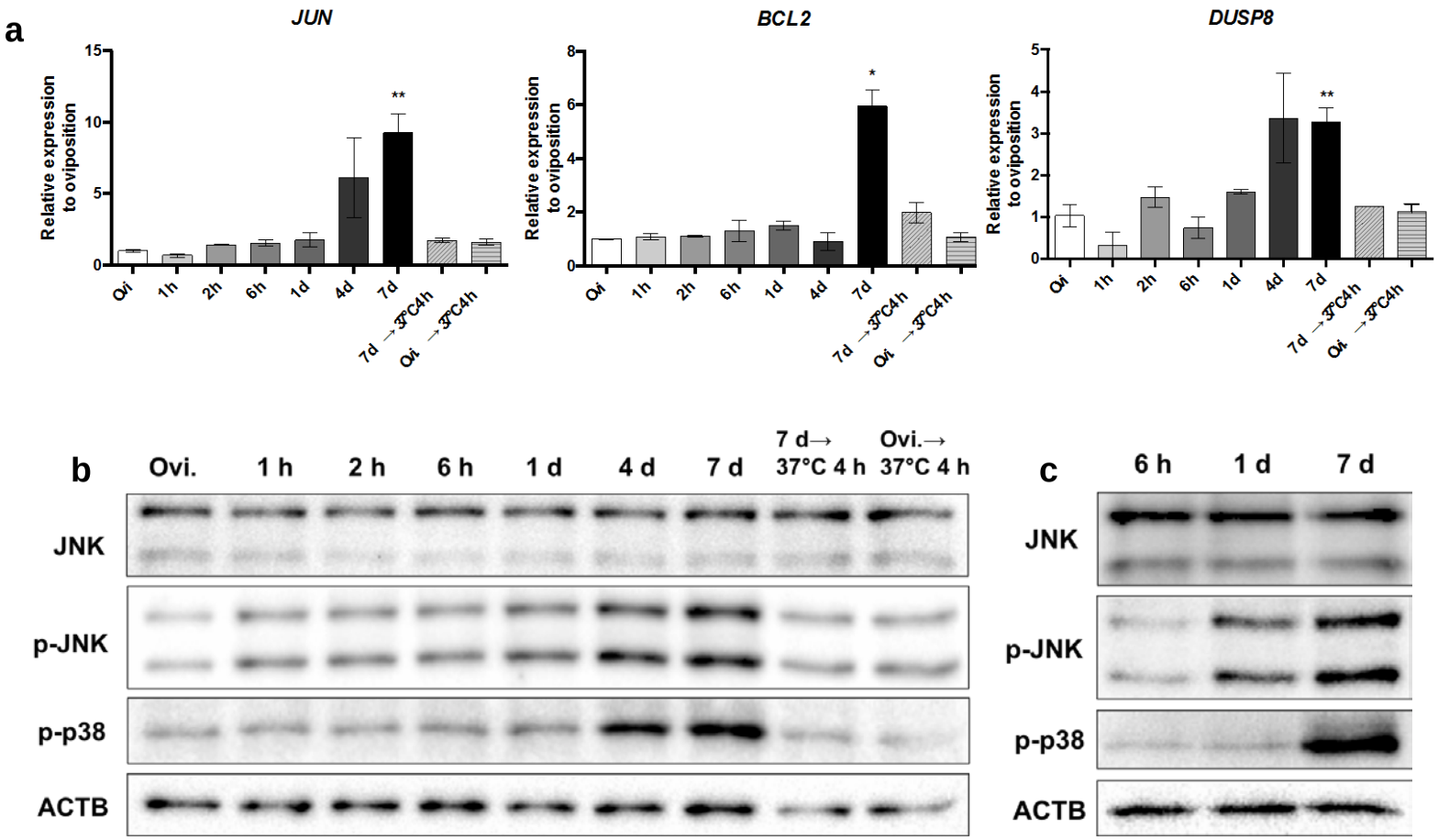

a
JUN
BCL2
DUSP8
Relative expression to oviposition
Ovi
1h
2h
6h
1d
4d
7d
7d →37°C4h
Ovi →37°C4h
b
Ovi.
1 h
2 h
6 h
1 d
4 d
7 d
7 d→ 37°C 4 h
Ovi.→ 37°C 4 h
JNK
p-JNK
p-p38
ACTB
c
6 h
1 d
7 d
JNK
p-JNK
p-p38
ACTB

Figure 3. Expression pattern of stress-activated protein kinase (SAPK) signaling related genes and proteins in the stage X embryo of chicken. Ovipositioned eggs were immediately taken or stored at 16°C for 1 hour, 2 hours, 6 hours, 1 day, 4 days, 7 days or incubated at 37°C for 4 hours for RNA isolation and observation of gene expression. a, qRT-PCR analysis of SAPK signaling related genes during storage. b, SAPK signaling protein expression during storage of chicken embryo. c, SAPK signaling protein expression during storage of duck embryo. For statistical analysis, the Student's t-test was used and values were compared to oviposition. *p value < 0.05, **p value < 0.01, ***p value < 0.001. Ovi., immediately ovipositioned embryo; h, hours of storage or incubation; d, days of storage.

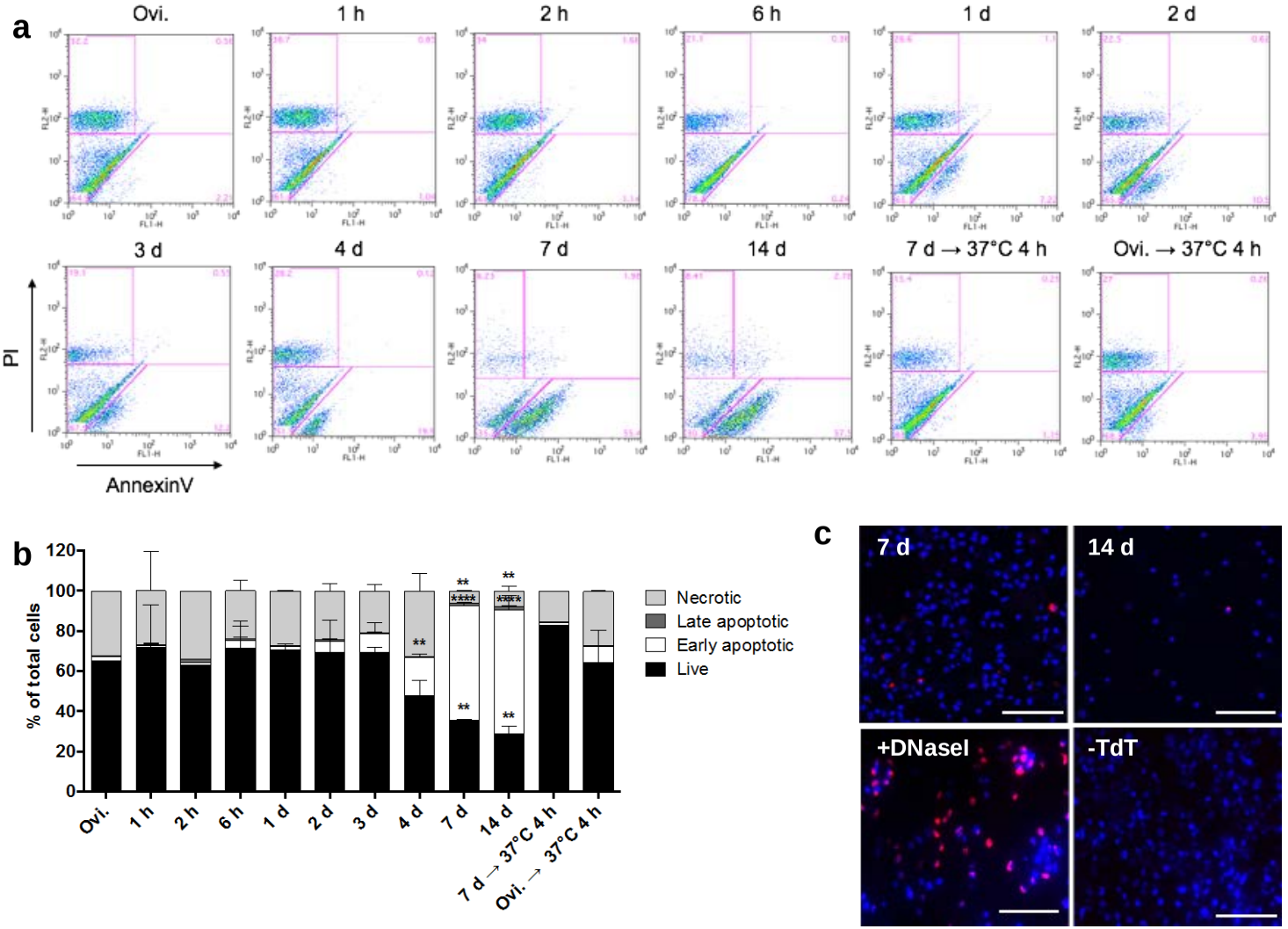
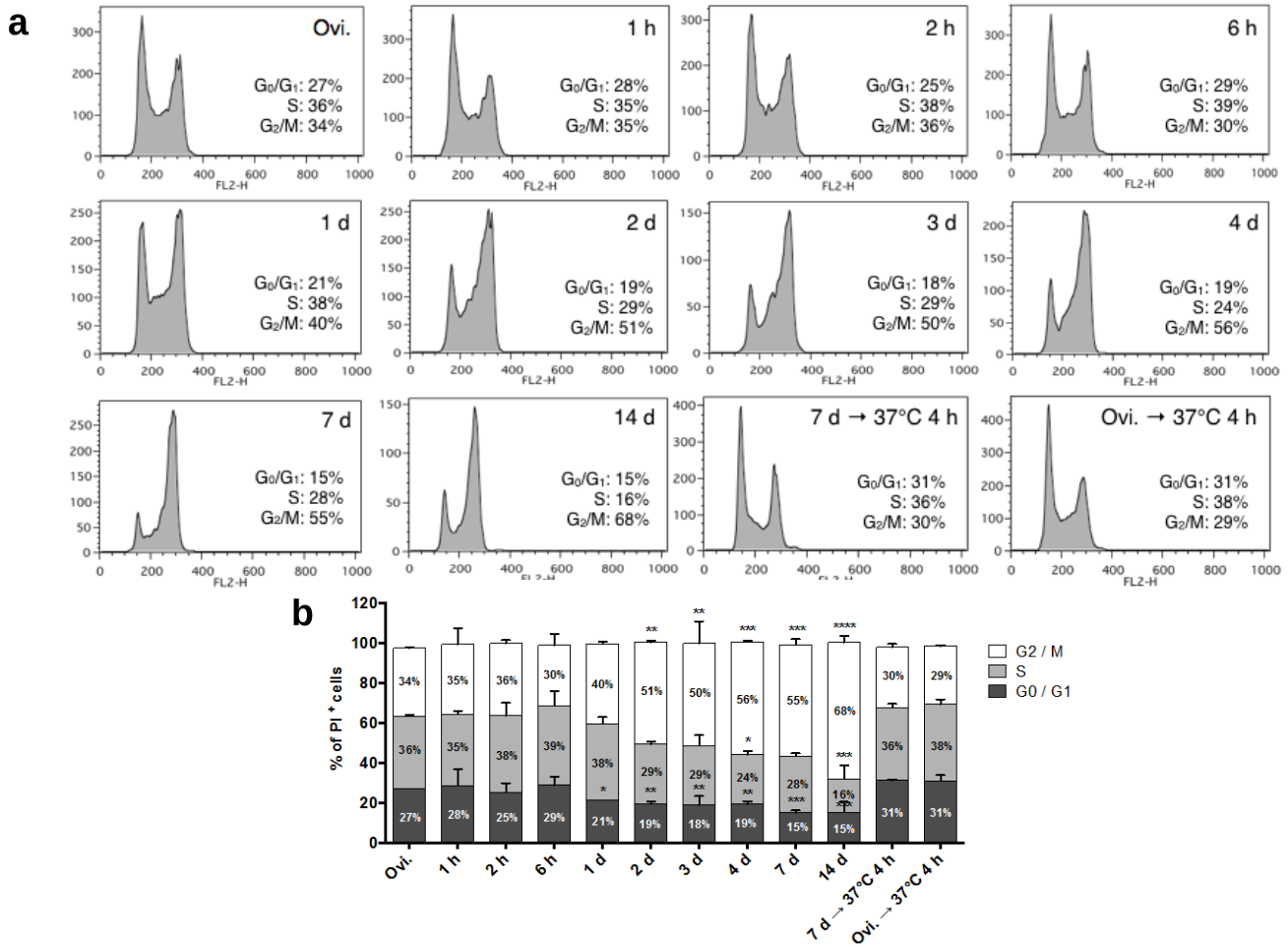

a
Ovi.
1 h
2 h
6 h
1 d
2 d
3 d
4 d
7 d
14 d
7 d → 37°C 4 h
Ovi. → 37°C 4 h
PI
AnnexinV
b
% of total cells
Necrotic
Late apoptotic
Early apoptotic
Live
c
7 d
14 d
+DNaseI
-TdT

Figure 4. Apoptosis is restricted to the early stage during chicken embryonic dormancy. Chicken blastoderm cells were observed for apoptosis using annexinV-PI staining and TUNEL assay. a, Dot plot of annexinV-PI staining of chicken blastoderm cells. b, Stacked bar graph of annexinV-PI stained blastodermal cells. For statistical analysis, one-way ANOVA test was used and values were compared to Ovi.. ****p value < 0.0001, **p value < 0.01. c, TUNEL assay of blastoderm cells storaged for 7 days or 14 days at 16 °C. Ovi., immediately ovipositioned embryo; h, hours of storage or incubation; d, days of storage; +DNaseI, DNase treatment for DNA breakage as positive control; -TdT.,removal of TdT enzyme as negative control. Scale bar is 100 μm.


a
Ovi.
G0/G1: 27%
S: 36%
G2/M: 34%
1 h
G0/G1: 28%
S: 35%
G2/M: 35%
2 h
G0/G1: 25%
S: 38%
G2/M: 36%
6 h
G0/G1: 29%
S: 39%
G2/M: 30%
1 d
G0/G1: 21%
S: 38%
G2/M: 40%
2 d
G0/G1: 19%
S: 29%
G2/M: 51%
3 d
G0/G1: 18%
S: 29%
G2/M: 50%
4 d
G0/G1: 19%
S: 24%
G2/M: 56%
7 d
G0/G1: 15%
S: 28%
G2/M: 55%
14 d
G0/G1: 15%
S: 16%
G2/M: 68%
7 d → 37°C 4 h
G0/G1: 31%
S: 36%
G2/M: 30%
Ovi. → 37°C 4 h
G0/G1: 31%
S: 38%
G2/M: 29%
FL2-H
b
% of PI + cells
G2 / M
S
G0 / G1
Ovi.
1 h
2 h
6 h
1 d
2 d
3 d
4 d
7 d
14 d
7 d → 37°C 4 h
Ovi. → 37°C 4 h

c

Figure 5. The cell cycle is arrested at the G_2/M phase during chicken embryonic dormancy. Chicken blastoderm cells were isolated and stained with propidium iodide (PI) for flow cytometry analysis of cell cycle status. a, Flow cytometric plot of PI stained chicken blastoderm cells. b, Stacked bar graph displaying percentage of blastoderm cells in each cell cycle phase. For statistical analysis, one-way ANOVA test was used and values were compared to Ovi.. ****p value < 0.0001, ***p value < 0.001, **p value < 0.01, *p value < 0.05. c, Western blot of phosphorylated histone H3 in the blastoderm during storage. Ovi., immediately ovipositioned embryo; h, hours of storage or incubation; d, days of storage.

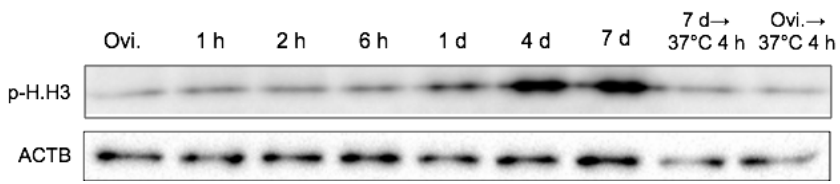


Table 1. Primer sequences for qRT-PCR

Gene symbol	Accession number	Size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
HSPA5	NM_205491.1	155	TTGCCAATGACCAGGGGAAC	ATTCCAGGTGCGGCCTATGA
ATF6	XM_422208.5	201	GACCATGCCTTACACAGACAC	GACATCTTTGGCCTTCTGGTC
MBTPS2	XM_425566.5	226	CAACCGGCAGTTCTACAACTG	CAGCTGGCTGACAGGCAA
DDIT3	XM_015273173.1	232	GAGGACAAAGCGGAAGCGT	GAAGCCATCAGTCCATGCCA
JUN	NM_001031289.1	204	CCCGGTGTATGCCAATCTCA	TCAATAGGGGACAGGGGAGG
DUSP8	XM_001232892.3	202	TTGTGTTGGTGTGCCTCAGT	AAAGTTGGGGTTGCCCAGAT
BCL2	NM_205339.2	304	TACCTGCTTACACTTAGGAAGG	ATGACTATGATGCGATGGCACG

Discussion

The avian embryo has a unique physiology, which could suspend development after oviposition until optimal incubation conditions are met, and this process is known as “cold torpor” (Ewert, 1991). The egg internal temperature measurement indicate that temperature drop occurs within 180 minutes post-oviposition at 16ºC, although the time will vary depending on the ambient temperature. The embryo under cold torpor is known to regulate various molecular processes, which is mainly related to stress responses.

According to the up-regulated processes from the transcriptome analysis, the extent of ER stress related gene and protein expression was observed. The up-regulation of *HSPA5* during storage indicated increase of the thermal stress response, resulting in activation of the apoptotic process that could be suggested from the up-regulation of *DDIT3* after 7 days of storage at 16ºC (Oyadomari and Mori, 2004). This corresponds to the increased phosphorylation of IRE1 during storage, as it is an ER stress effector that is especially known to activate SAPK signaling (Chen and Brandizzi, 2013; Urano et al., 2000). The SAPK signaling is known to be activated in response to various cellular stress stimuli, and the signaling of JNK and p38 MAPK pathways result in the activation of downstream transcription factors, which regulates various cellular processes as transcription or apoptosis (Johnson and Nakamura, 2007). It was shown that the amount phosphorylation of JNK and p38 is elevated after 7 days of storage at 16ºC, while at the same time, the amount of downstream transcription factor expression increased. However, regardless of the

duration of storage, when the embryo was incubated at 37ºC, the expression of both ER stress and SAPK signaling related genes and proteins decreased. This implicates that the stress related processes are inactivated after the embryonic dormancy ends.

Once ER stress or SAPK signaling is active, it induces the activation of apoptotic genes, resulting in the promotion of cellular death. Observation of apoptotic status in the dormant chicken embryo has suggested that the amount of live and necrotic cells decreases during storage, while the number of early apoptotic cells increase. Early apoptotic cells has hallmarks as representation of phosphatidylserine (PS) on cell surface with intact cellular membrane, which could be contrasted to the late apoptotic cell that has lost membrane integrity (Poon et al., 2010). Interestingly, the number of late apoptotic cells was maintained by a low proportion lower than 2% of total cells, indicating little damage in cellular integrity. The TUNEL assay confirmed that there was handful amount of DNA double strand breakage. However, after incubation at 37ºC, the apoptotic status became similar to the ovipositioned period, showing that the early apoptotic cells could reverse to live cells. It was previously demonstrated that cells that are shortly exposed to apoptotic stimuli could reverse the early apoptotic status to normal status with the removal of the stress stimulus (Tang et al., 2012). Similarly in the avian blastoderm, recovery from mild cellular death progress is capable, and compared to other types of mammalian cells the blastoderm could endure longer exposure to cold stress exposure for at least 7 days.

A representative characteristic of embryonic dormancy is the change of cellular status, which includes the quiescence of cell proliferation and cell cycle (Lopes et al., 2004). It is wildly known that mitotic arrest occurs at the G_0/G_1 phase in most mammalian embryos, which is tightly controlled by specific cell cycle inhibitors (Padilla and Ladage, 2012; Podrabsky and Culpepper, 2012). However, in the avian blastoderm, it was shown that the mitotic arrest occurs at the G_2/M phase, which gradually starts within 2 days of storage. However, after incubation at 37ºC, the blastoderm cells could escape mitotic arrest and progress through the cell cycle. This is similar to the lower order species, *Caenorhabditis elegans*, which is known to arrest cell cycle at the M phase during embryonic dormancy under the control of various cyclins and cyclin dependent kinases (Padilla and Ladage, 2012). Nonetheless, the factors that cause the cell cycle arrest in avian embryo are still unknown.

Collectively, compared to other types of mammalian cells, the avian blastoderm has a stress-tolerant ability that enables to endure cold stress after the oviposition until adequate developmental conditions are met. This study could provide general molecular phenomenon that occurs in the avian blastoderm during dormancy, which could be classified to activation of ER stress and SAPK signaling, increase in early apoptotic cells and arrest of cell cycle in G_2/M phase. However, the exact mechanisms and molecular signaling that are responsible for this phenomenon is still unexcavated and requires further examination. Further studies should be required to address the mechanisms underlying the regulation of cellular processes during embryonic dormancy of avian species.

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SUMMARY IN KOREAN

조류의 배아는 방란 이후 일정기간 모체의 난관온도 보다 낮은 온도의 환경에 보관되어질 수 있다. 배아 발달 중 낮은 온도 노출에 대하여 저항성이 있음을 “cold torpor”라고 일컫는데, 이는 배아가 발달하는 중에 외부로 부터 오는 저온 스트레스에 저항하기 위하여 주로 발달휴면기를 갖는 배아에서 나타난다. 대부분의 조류는 clutch 의 마지막 알이 방란되어질때 동시에 모든 알을 품는데, 이러한 습성은 clutch 에서 마지막으로 방란된 알이 바로 품어지게되는데 반해, 처음으로 방란된 알은 장기간 저온에 노출되어지게 한다. 이런 조류의 휴면기간동안의 변화에 대하여, 난내 pH 증가나 난백 및 난황 무게 감소 등 생리적인 변화는 많은 연구가 진행되었지만, 아직 조류 배아의 휴면기 동안의 분자생물학적인 변화에 대한 연구는 미흡한 상황이다. 그리하여 본 연구에서는 방란 이후 휴면기의 조류 배아 내에서 일어나는

분자생물학적인 변화를 규명하고 방란 이후의 기간동안 조류 배아가 항상성을 유지하는 기작을 연구하였다.

발생 휴면 기간의 조류 배아 내에서 일어나는 분자유전학적인 기작들을 알아보기 위하여, RNA sequencing 을 통하여 증감하는 기작을 알아본 결과, endoplasmic reticulum (ER) stress, stress-activated protein kinase (SAPK) signaling 관련 유전자의 발현이 방란 7 일 이후에 유의적으로 증가하며, 핵산 대사과정에 관련한 유전자의 발현은 방란 7 일 이후에 유의적으로 감소하는 것으로 나타났다.

RNA sequencing 결과를 바탕으로, 실제로 조류의 배아 내에서 휴면기 동안 일어나는 유전자 및 단백질 발현을 알아보았다. 그 결과 ER stress 에 관련한 유전자의 발현이 모두 방란 이후 7 일에 유의적으로 발현되는 것을 확인하였다. 또한 ER stress 의 매개 단백질 중 하나인 IRE1 의 인산화가 방란 이후 1 일과 7 일에 증가하는 것을 확인함으로써, 방란 이후에 ER stress 의 활성 정도가 증가하는 것을 알 수 있었다. ER

stress 에 더하여 SAPK signaling 또한 관련 유전자가 방란 이후 7 일에 유의적으로 높게 발현되는 것을 확인하였으며, mitogen-activated protein kinase (MAPK) 인 p38 과 JNK 의 단백질 인산화 정도가 방란 이후 7 일에 높게 나타나는 것을 확인하였다. 이로써, 우리는 조류 배아의 발생 휴면기 동안 ER stress 와 SAPK signaling 이 활성화 됨을 알 수 있었다.

ER stress 와 SAPK signaling 의 활성도는 세포자멸에 밀접한 연관이 있다고 알려져있다. 방란 이후의 휴면 기간 동안 조류 배아에서 일어나는 세포자멸의 정도를 측정한 결과, 방란 이후 시간이 지남에 따라 Live cell 과 necrotic cell 의 수는 줄어드는 반면에, early apoptotic cell 의 수는 증가함을 알 수 있었다. Early apoptotic cell 은 cell membrane 에 손상이 일어나지 않고 membrane 의 phosphatidyl serine (PS)가 외부로 노출되어있는 상태를 말하는데, 이는 초기 세포사멸단계의 세포에서 주로 나타난다. 이에 반하여 late apoptotic cell 의 정도는 낮은 수준 (≤2%)으로 유지되었는데, 이것은 TUNEL assay 로도 DNA 이중가닥

절단이 매우 낮은 수준으로 일어나는 것을 확인함으로써, 방란 이후의 휴면 기간 동안에 DNA 의 손상이 적은 수준으로 일어나며, 최대 14 일의 휴면기간에도 손상이 적다는 것을 알 수 있었다. 하지만 휴면 기간을 끝내고 37°C 에서 4 시간 동안 발생을 유도한 결과 세포자멸이 현저히 떨어지고 방란 직후의 수준으로 live cell 이 늘어남을 알 수 있었다. 이로써 우리는 방란이후의 저온 스트레스에도 조류의 배아는 적절한 온도 조건이 갖추어지면 발생이 가능하며, 휴면기간동안 늘어난 초기 세포자멸단계의 세포들이 줄어듦을 알 수 있었다.

마지막으로 우리는 휴면 기간 동안 조류 배아의 세포 주기를 관찰하였다. 그 결과 방란 이후 시간이 지남에 따라 최소 2 일 이후 부터 배아 세포가 G_2/M 단계에 정지해 있다는 것 을 알 수 있었다. G_2/M 단계에 정지해 있는 세포의 수는 방란 이후 2 일에 50%에서 방란 이후 14 일에는 68%까지 증가하였다. 하지만 이 또한 37°C 에서 4 시간 동안 발생을 유도할 시에는 방란 초기의 상태로 돌아오는 것 을 알 수 있었다.