Function and regulation of minor zygotic gene activation in mouse embryos

(受精後における最初期の遺伝子発現の機能
および制御機構の解明)

阿部　健一郎

Function and regulation of minor zygotic gene activation in mouse embryos

Kenichiro Abe

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Department of Integrated Bioscience

Graduate School of Frontier Science

The University of Tokyo

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Summary

In sexual reproductive animals, the life begins from fertilized oocytes, which develop into the multicellular organism. In the process of development, the pattern of gene expression is dynamically changed. Prior to fertilization, the oocyte genome is not transcribed and this transcriptionally silent state is maintained for a while after fertilization. In mice, the gene expression is initiated at the mid-1-cell stage and the transcriptional activity is dramatically increased at the 2-cell stage. This activation of gene expression is called as zygotic gene activation (ZGA). The expression pattern in ZGA changes during DNA replication at the 2-cell stage. Previous studies using reporter genes revealed that transcription is stimulated by enhancers in the late 2-cell stage embryos but not 1-cell stage ones and that this enhancer independent gene expression was still observed at the late 2-cell stage when the DNA replication was inhibited. The pattern and regulation of gene expression is thus dramatically altered during the second round of DNA replication. Therefore, to distinguish the ZGA before and after the second round DNA replication, the former and later is called as minor and major ZGA, respectively. Although the function and regulatory mechanism of major ZGA has been well elucidated, those of minor ZGA have not been clarified.

In chapter I, to explore the biological function of minor ZGA, I temporarily inhibited the transcription during minor ZGA using 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a reversible inhibitor of RNA polymerase II, and analyzed the effect of the treatment with DRB on the preimplantation development. The majority of embryos treated with DRB were arrested at the 2-cell stage, uncovering that minor ZGA is essential for the development. To

further investigate the role of minor ZGA in the regulation of preimplantation development, I analyzed the gene expression using RNA sequencing in the 2-cell stage embryos after the removal of DRB. The results revealed that the temporal inhibition of transcription during minor ZGA resulted in the failure in major ZGA which is essential for the cleavage into the 4-cell stage. Thus, minor ZGA plays an essential role for occurrence of major ZGA.

In chapter II, to find a key player of minor ZGA for embryonic development, I surveyed the transcription from intergenic regions, since a recent study suggested that the transcripts from protein-coding genes have no function at the 1-cell stage: they are not translated into functional proteins at this stage. The analysis of RNA sequencing revealed that intergenic regions were pervasively transcribed during minor ZGA, although the pervasive transcription was repressed during the second round of DNA replication. The analysis to find the upstream elements regulating the pervasive transcription suggested that G/C-rich regions are involved in the regulation of this type of transcription. The pervasive occurrence of intergenic transcription led me to the hypothesis that a non-coding RNA transcribed from the intergenic regions is involved in the regulation of preimplantation development. From RNA sequencing data and reverse transcription and polymerase chain reaction using preimplantation embryos and adult tissues, I found 4 species of RNAs which were transcribed from intergenic regions and expressed specifically in early preimplantation embryos. Reduction of the expression of these intergenic RNAs using LNA-DNA gapmers caused the developmental arrest before the blastocyst stage. These results suggested that intergenic non-coding RNAs which are transcribed during minor ZGA are involved in the regulation of preimplantation development.

Taken together, I provided the evidence that minor ZGA has a critical function for preimplantation development through the activation of the subsequent major ZGA. This transcriptional cascade from minor to major ZGA may be regulated by intergenic RNAs which are transcribed during minor ZGA.

General introduction

Life arises from a single cell, *i.e.* a zygote. The development from zygotes to the adult multicellular organisms is achieved through the changes in gene expression patterns. Before fertilization, mouse oocytes express the genes, including oocyte-specific genes, *e.g. Zp3* and *c-Mos*, in a specific pattern. During their growth, mRNA is highly stable and accumulated in the cytoplasm as maternal mRNAs (Schultz, 1993; Yu et al., 2003). However, their transcription ceases near the time when the oocytes grow into their full size and the transcriptionally silenced state continues during meiosis and even after fertilization (Moore et al., 1974; Aoki et al., 1997; Abe et al., 2010). Therefore, the accumulated maternal mRNA supports the meiosis progression and the development of embryos for a while after fertilization. In the earlier studies, the initiation of transcription from zygotic genes, which was designated as zygotic gene activation (ZGA), was considered to occur at the 2-cell stage but not at 1-cell stage in mice, because gel electrophoresis analyses of the proteins incorporating [^{35}S]methionine showed that the protein synthesis pattern did not change until 2-cell stage after fertilization. However, soon after cleaved into 2-cell stage, a few proteins which had not been observed in the oocytes or 1-cell stage embryos appeared (van Blerkom and Brockway, 1975). Addition of α-amanitin, a potent inhibitor of RNA polymerase II (Pol II), prevented or reduced the appearance of these proteins (Levey et al., 1978). Therefore, these proteins, whose molecular weight was ~70 kd, were considered as the products derived from the transcripts in ZGA and designated as transcription requiring complex (TRC) (Conover et al., 1991). The gel electrophoresis analysis of the newly synthesized proteins also revealed that the protein synthesis patterns dramatically

changed during S phase of the 2-cell stage, suggesting that the gene expression patterns grossly alters at this time (Flach et al., 1982; Bolton et al., 1984). Therefore, this burst of zygotic gene activation at mid to late 2-cell stage was designated as major ZGA to discriminate from minor ZGA transcribing mRNAs encoding TRC, the initial transcription from zygotic genome, at the early 2-cell stage (Flach et al., 1982). Although in these pioneering studies, the transcription from zygotic genes was not detected before 2-cell stage, the later studies using more sensitive assays, *i.e.* BrUTP incorporation and the expression of a plasmid reporter genes, revealed that the transcription from zygotic genes begins at the S phase of 1-cell stage (Matsumoto et al., 1994; Aoki et al., 1997). Therefore, it is now recognized that minor ZGA occurs between S phase of the 1-cell stage and G1 phase of the 2-cell stage.

The second round of DNA replication is involved in the transition of the gene expression patterns from minor to major ZGA. Two-dimensional gel electrophoresis and mRNA differential display identified the markers for minor ZGA, *i.e.* TRC, *Hsp70* and *Eif1a* (Bensaude et al., 1983; Conover et al., 1991; Davis et al., 1996). The expression of these minor ZGA markers were repressed at the late 2-cell or 4-cell stage but remained high when the second round of DNA replication was inhibited by aphidicolin which is the inhibitor of DNA polymerase (Ohashi et al., 1978). These reports suggested that minor ZGA is repressed by the second round of DNA replication, resulting in the transition of the gene expression pattern from minor ZGA into major ZGA. In addition, it has also been shown that the second round of DNA replication is implicated in the changes in the regulation of gene expression during the transition from minor ZGA into major one. Generally, enhancers activate the repressed promoters. However, the activation of promoters by enhancers was not observed before 2-cell stage after

fertilization (Wiekowski et al., 1991; Majumder et al., 1993; Majumder and DePamphilis, 1995; Lawinger et al., 1999). The expression of reporter gene was stimulated by an enhancer in 2-cell stage embryos, but not at the 1-cell stage. When the second round of DNA replication was inhibited by aphidicolin, the expression of reporter gene was not stimulated by an enhancer at even the late 2-cell stage (Majumder et al., 1993; Henery et al., 1995; Majumder et al., 1997; Forlani et al., 1998). The second round of DNA replication thus changes the regulation of gene expression from the enhancer-independent manner in minor ZGA into the enhancer-dependent one in major ZGA.

The requirement for enhancers seems to be regulated by the chromatin structure in the early embryos. In the late 2-cell stage embryos treated with butyrate, a histone deacethylase inhibitor that loosens the chromatin structure, the expression of injected reporter genes were actively expressed by a promoter and not stimulated by an enhancer as in 1-cell stage embryos and the late 2-cell stage embryos whose DNA replication had been inhibited by aphidicolin (Majumder et al., 1993; Majumder and DePamphilis, 1995; Majumder et al., 1997; Lawinger et al., 1999). These results suggested that the loosened chromatin structure is involved in the enhancer-independent transcriptional regulation in minor ZGA.

Minor ZGA is the first gene expression after fertilization, but this biological function has not been clarified. It has been thought that major ZGA is required for the embryonic development but not minor ZGA, because the embryos were cleaved into 2-cell stage and then arrested at the G2 phase of 2-cell stage when they had been cultured in the presence of α-amanitin from the time just after fertilization (Warner and Versteegh, 1974). However, this experiment is insufficient to prove that minor ZGA is

not essential for embryonic development, since it is possible that 1-cell stage embryos could accomplish the first cell cycle by using the proteins translated from the accumulated maternal mRNAs and that zygotic transcripts derived from minor ZGA are required for the progression of the second cell cycle. To clarify the biological function of minor ZGA, it is required to inhibit only minor ZGA but not subsequent transcriptions.

Previous studies have suggested the specific mechanisms regulating minor ZGA. However, most of those results were obtained by reporter gene assays using exogenous promoters and enhancers. To investigate the transcriptional regulation in minor ZGA, the analysis for the endogenous *cis*-acting elements involved in minor ZGA would be essential. However, although several reports showed the results of the microarray analysis for the changes in gene expression patterns during preimplantation development and suggested the *cis*-acting elements regulating the gene expression in major ZGA at the 2-cell stage and thereafter (Hamatani et al., 2004; Wang et al., 2004), little is known about such elements in minor ZGA because the small number of transcribed regions are known in minor ZGA. The reason comes from the fact that the amount of mRNA transcribed in minor ZGA is small and masked by a large amount of accumulated maternal mRNA in the analysis of microarray. To analyze endogenous *cis*-elements, it is required to sensitively detect a small amount of zygotic transcripts in minor ZGA.

Recent RNA sequencing (RNA-seq) technology is known as alternative approach for microarray on genome wide transcriptome analysis. RNA-seq is more precise and quantitative method than microarray (Xiong et al., 2010). Therefore, this method is promising approach to detect zygotic transcripts whose amounts are quite lower than

maternal ones. Furthermore, RNA-seq technology allows the detection of intergenic transcripts. Previous microarray analyses did not carry comprehensive information on gene expression profiles in 1-cell stage embryos, because they used the probes which targeted annotated genes only. Emerging evidence has revealed that a long non-coding RNA transcribed from intergenic regions plays important role in the regulation of gene expression in the various types of cell (Loewer et al., 2010; Zhao et al., 2010; Cabili et al., 2011; Cesana et al., 2011; Grote et al., 2013; Klattenhoff et al., 2013). However, little is known about these RNAs transcribed in minor ZGA. RNA-seq would be the best method to obtain the information about them.

In this thesis, I investigated the function and the mechanism regulating minor ZGA. In chapter I, to elucidate the biological function of minor ZGA, I inhibited only minor ZGA using DRB, a reversible inhibitor of RNA polymerase II (Zandomeni et al., 1986; Raju et al., 1991), and examined the effect of inhibition of minor ZGA in the preimplantation development. I also analyzed the gene expression after the temporal inhibition of minor ZGA to examine its involvement in the regulation of major ZGA. In chapter II, to find an functional element of minor ZGA, I surveyed an intergenic RNA specifically transcribed during minor ZGA using RNA-seq and investigated their roles in the preimplantation development. In addition, I also found the unique transcription feature of minor ZGA by whole genome transcriptome analysis using RNA-seq. To explore the mechanism regulating this transcription during minor ZGA, I examined the *cis*-elements of transcribed regions.

Fig. 1

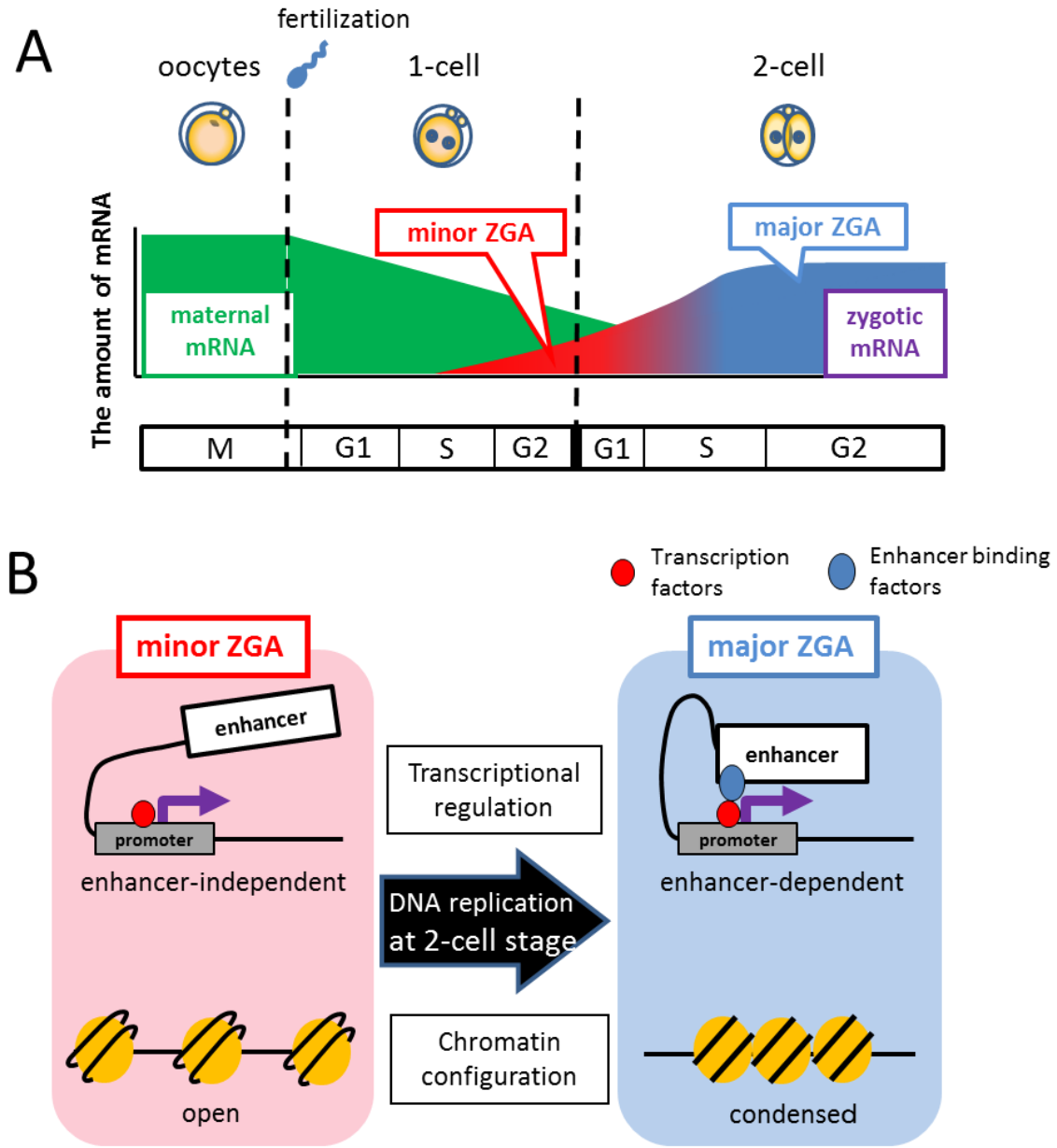

Figure 1. Differences of regulatory mechanism between minor ZGA and major ZGA. (A) The timing of minor- and major ZGA before and after fertilization. Minor ZGA occurs between S phase of 1-cell stage and G1 phase of 2-cell stage. The gene expression changes from minor ZGA to major ZGA during S phase of 2-cell stage. (B) Changes in the mechanism of transcriptional regulation during ZGA. The opened chromatin structure contributes to the enhancer independent transcriptional regulation during minor ZGA. However, the chromatin is condensed during the second round of DNA replication, resulting in the requirement of enhancers for active transcription during major ZGA.

Chapter I

Functional analysis of minor ZGA

Result

Inhibition of the transcription during minor ZGA by DRB

To investigate the involvement of minor ZGA in the regulation of preimplantation development, I used DRB to inhibit the transcription during minor ZGA. Since DRB is a reversible inhibitor for RNA polymerase II dependent transcription, the transcriptional activity is recovered after its removal (Raju et al., 1991), implying that DRB could inhibit the transcription solely during the phase of minor ZGA by removing the drug from a medium soon after the termination of minor ZGA phase. Since the transition from the phase of minor ZGA to that of major ZGA occurs during the second round of DNA replication (Flach et al., 1982; Bolton et al., 1984), DRB should be removed at the time of the occurrence of DNA replication. Therefore, I first investigated the time of initiation of DNA replication at the 2-cell stage by the incorporation of BrdU. I found that most embryos reached to S phase at 20 h after insemination (Fig. 2A). Since previous study showed that minor ZGA starts around the mid-S phase of 1-cell stage (Aoki et al., 1997), I decided to treat the embryos with DRB from G1 phase of 1-cell stage (4 h after insemination) until G1 phase of 2-cell stage (20 h after insemination). To determine the minimum effective concentration of DRB, I examined the transcriptional activity by the incorporation of BrU in the 1-cell stage embryos treated with various concentrations of DRB (0, 20, 40, 60, 80 and 100 μM). The transcriptional activity decreased along with the increase in the concentration of DRB until 80 μM, and the inhibitory effect on the transcription plateaued over this concentration (Fig. 2B). Thus, I decided to treat the embryos with 80 μM of the drug as its minimum effective concentration on the inhibition of minor ZGA.

To verify the reactivation of the transcription after the removal of DRB, I examined the transcriptional activity by the incorporation of BrU in the 1-cell and 2-cell stage embryos during and after the treatment with DRB. In the absence of DRB, all embryos exhibited active transcription. However, the embryos treated with DRB showed no or little transcriptional activity (Fig. 2C). When the embryos which had been treated with DRB were transferred to the medium without DRB, the transcription was activated 1 h after the transfer. In addition, I also observed the phosphorylation on C-terminal domain (Ser2P) of Pol II which is associated with transcriptional elongation, since DRB represses the transcription through inhibiting the activity of protein kinases that target Pol II. Immunocytochemistry with the antibody against Ser2P revealed that the signal of fluorescence was not detected in the embryos when they were treated with DRB and then appeared 1 h after the transfer to the medium without DRB (Fig. 2D), indicating again that the transcription did not occur in the embryos treated with DRB and started soon after the removal of the drug. To further ascertain the inhibitory effect of DRB on transcription, I conducted RT-PCR in the 1-cell stage embryos treated with DRB to examine the expression level of the genes which are transcribed during minor ZGA. I confirmed that the expression levels of all genes examined were markedly reduced by the treatment with DRB (Fig. 2E).

Involvement of minor ZGA in the regulation of preimplantation development

The embryos were transferred to the medium containing DRB 4 h after insemination, and then they were moved to DRB-free medium 20 h after insemination and cultured until 96 h. In the control embryos which had not been treated with DRB, 98% of them cleaved to 3-4 cell or 6-8 cell stages. Thereafter, 94% and 92% of the

embryos developed to the molura and blastocyst stages 72 h and 96 h after insemination, respectively (Fig. 3A and 3B). However, most of the embryos treated with DRB were arrested at the 2-cell stage 46 h after insemination. At 96 h, more than 60 % of them were still arrested at 2-cell stage and only 10 % of the embryos reached the blastocyst stage. To examine the phase of cell cycle at which those embryos were arrested at the 2-cell stage, I examined the occurrence of DNA replication in DRB-treated embryos by the incorporation of BrdU 26 h after insemination. They incorporated BrdU as was observed in the control embryos (Fig. 3C). Since the nuclei was clearly observed in the arrested embryos (data not shown), they had not entered into M phase. Therefore, the embryos were arrested at G2 phase of the 2-cell stage.

To assess the effect of the inhibition of major ZGA in the preimplantation development, the embryos were treated with DRB from 26 to 42 h after insemination. 70% of them cleaved to 3-4 cells, but 30% of them remained at the 2-cell stage (Fig. 3A). However, the percentage of the embryos at 2-cell stage decreased to 6%, and that of the embryos which had developed beyond 2-cell stage increased to 93% 72 h after insemination. At 96 h, more than 40% and 30% of the embryos reached the morula and blastocyst stage, respectively. Thus, the rate of development was retarded but the developmental potential remains by the temporal inhibition of major ZGA, although the potential was completely lost by the inhibition of minor ZGA. These results suggest that the timely occurrence of minor ZGA but not major one is essential for the preimplantation development.

Effect of temporal inhibition of minor ZGA on the gene expression at the late 2-cell stage

To investigate the cause of 2-cell-arrest in the embryos in which minor ZGA were inhibited with DRB, I examined the gene expression at the late 2-cell stage after the removal of DRB. Firstly, I measured the transcriptional activity by the incorporation of BrU. After the removal of DRB, the transcriptional activity drastically increased. It was much higher than that in the control embryos without the treatment with DRB until 29 h after insemination. However, the activity started decreasing from 29 h, and it became to be lower than the control embryos 32 h and continued to decrease until 96 h after insemination (Fig. 4). These results suggested that the Pol II paused around promoter regions by DRB was rapidly reactivated soon after the removal of DRB, and the genes expressed during this period would make a detrimental effect on the subsequent gene expression: An aberrant gene expression different from the programmed one would occur after the removal of DRB.

To investigate the effect of inhibition of minor ZGA on the gene expression after the removal of DRB, I conducted RNA sequencing for the DRB-treated 2-cell stage embryos 12 h after the removal of DRB (32 h after insemination) and compared the expression levels of genes between DRB-treated and -untreated embryos. In this analysis, it was useful to extract the genes that are transcribed during minor and major ZGA and then examine the effect of temporal inhibition of minor ZGA in their expression. Therefore, I classified the genes by the changes of their expression levels at the oocyte, 1-cell and 2-cell stages using a k-means nonhierarchical clustering method (Saeed et al., 2003). When the genes were categorized into five classes, cluster I consisted of the genes whose expression levels did not change after fertilization (between oocyte and 1-cell stage) but increased at the 2-cell stage, indicating that this cluster mostly contained the genes transcribed during major ZGA but not minor ZGA

(Fig. 5A). The genes classified into cluster II decreased their expression levels after fertilization but increased at the 2-cell stage, which indicates that this cluster also contains the genes transcribed during major ZGA but not minor ZGA as well as cluster I. The cluster III consisted of the genes which continuously decreased their expression levels after fertilization and the cleavage into 2-cell stage. The genes in this class did not seem to be activated during any of minor and major ZGA. Cluster IV mostly contains the genes whose expression levels increased after fertilization and then further increased at the 2-cell stage, indicating that they are transcribed during both of minor and major ZGA. Finally, the genes in cluster V increased their expression levels after fertilization and decreased at the 2-cell stage. The genes in this cluster seemed to be transcribed during minor ZGA but not major one. When the expression levels were compared between the embryos treated with and without DRB, more than half of the genes in cluster I and II, which were transcribed during only major ZGA but not minor one, decreased their expression levels after the removal of DRB (Fig. 5A). On the contrary, a large part of the genes in cluster IV and V, which were transcribed during minor ZGA, increased their expression after the removal of DRB. The similar results were obtained when the numbers of highly up-regulated and down-regulated genes were examined in each cluster (Fig. 5B). In cluster I and II, the numbers of down-regulated genes were higher than those of up-regulated ones. On the contrary, in cluster IV and V, the numbers of up-regulated genes were much higher. These results indicated that a large part of genes which are supposed to be transcribed during minor ZGA were activated after the removal of DRB, but that those transcribed during major ZGA were not sufficiently transcribed at the time (32 h after insemination) when major ZGA normally occurs. Therefore, it is possible that the deficient expression of genes which are

transcribed during major ZGA caused the arrest of DRB-treated embryos at 2-cell stage: it was shown that major ZGA is essential for 2-cell stage embryos to cleave into 4-cell stage (Warner and Versteegh, 1974). To address this hypothesis, I examined the expression of the genes which play important roles in the regulation of cell cycle in DRB-treated embryos. Because it has been supposed that the embryos treated with DRB were arrested at G2 phase of 2-cell stage (Fig. 3C), I focused on the genes which are involved in G2/M transition. I found that all of *Ccna2*, *Cdk1* and *Cdc25c*, whose encoding proteins are essential components of M-phase promoting factor and its activator (Hutchins and Clarke, 2004; Wang et al., 2011; Wolgemuth, 2011), belonged to cluster I (Fig. 6) and their expression was reduced by the treatment with DRB (Fig. 6). These results suggested that the reduction of the expression of these genes caused the developmental arrest in the DRB-treated embryos. Taken together, I suggest that the temporal inhibition of minor ZGA caused the deficient activation of the cell cycle-related genes which are set to be expressed during major ZGA, leading to the developmental arrest of DRB-treated embryos.

Discussion

Previous studies using micro array revealed that the pattern of gene expression is dynamically changed during preimplantation development (Hamatani et al., 2004; Wang et al., 2004; Kageyama et al., 2007). It is thought that this change is precisely controlled under a program of gene expression. However, it has not been clarified how strict timing of the expression at each developmental stage is required for the normal progression of the program. In the present study, I have shown that the temporal inhibition of minor ZGA caused the failure in major ZGA and the developmental arrest at the 2-cell stage, although a large number of genes which are supposed to be transcribed during minor ZGA were expressed after the removal of DRB (Fig. 5A and B). This result suggested that major ZGA requires the occurrence of minor ZGA at an appropriate time. On the contrary, when major ZGA was inhibited by the treatment with DRB, 70% of embryos successfully developed to the morula and blastocyst stages (Fig. 3A), implying that major ZGA occurred after the removal of DRB. Taken together, my results suggested that the gene expression program is strictly regulated by the stage specific manner during minor ZGA but not major one.

During the treatment with DRB, the transcriptional machineries containing Pol II possibly remained to be bound to the promoters of the genes which were supposed to be transcribed during minor ZGA, because DRB represses transcriptional elongation through the inhibition of phosphorylation of Pol II (Chodosh et al., 1989; Dubois et al., 1994; Larochelle et al., 2012). Therefore, the transcriptional machineries bound to around promoter regions seemed to rapidly resume the transcriptional elongation after the removal of DRB, resulting in the activation of genes which were supposed to be

transcribed during minor ZGA but not major ZGA at the 2-cell stage. Supporting this hypothesis is that the transcriptional activity was markedly increased soon after the removal of DRB (Fig. 4). Since most of Pol II molecules would be stuck around the promoter regions without moving away to transcribe the coding regions during the treatment with DRB, the burst of transcription might occur by the concurrent activation of those stuck Pol II after the removal of DRB.

The temporal inhibition of minor ZGA caused the developmental arrest at G2 phase of the 2-cell stage (Fig. 3C). Although a group of genes which are supposed to be transcribed during minor ZGA were activated soon after the removal of DRB (cluster IV and V in Fig. 5A and B), a majority of the genes transcribed during major ZGA were not activated (cluster I and II in Fig. 5A and B). Therefore, it seemed that the developmental arrest was due to the deficient expression of the genes which are essential for the progression of cell cycle from G2 to M phase. Indeed, the expressions of various cell cycle-related genes were reduced by the treatment with DRB (Fig. 6). Alternatively, it is also possible that the expression of the genes of minor ZGA at the late 2-cell stage had a detrimental effect on the regulation of cell cycle progression. A recent study reported that most of genes are transcribed at the 1-cell stage. If these transcripts are translated into proteins, such an unregulated expression of the proteins would have detrimental effects on the progression of cell cycle. However, the majority of mRNAs transcribed at the 1-cell stage were not translated into functional proteins because they were not spliced (Yamamoto et al., 2012). When the genes which are supposed to be transcribed during minor ZGA were activated after the removal of DRB, they would be spliced and translated into proteins which might have an adverse impact on the regulation of cell cycle at the late 2-cell stage.

Fig. 2

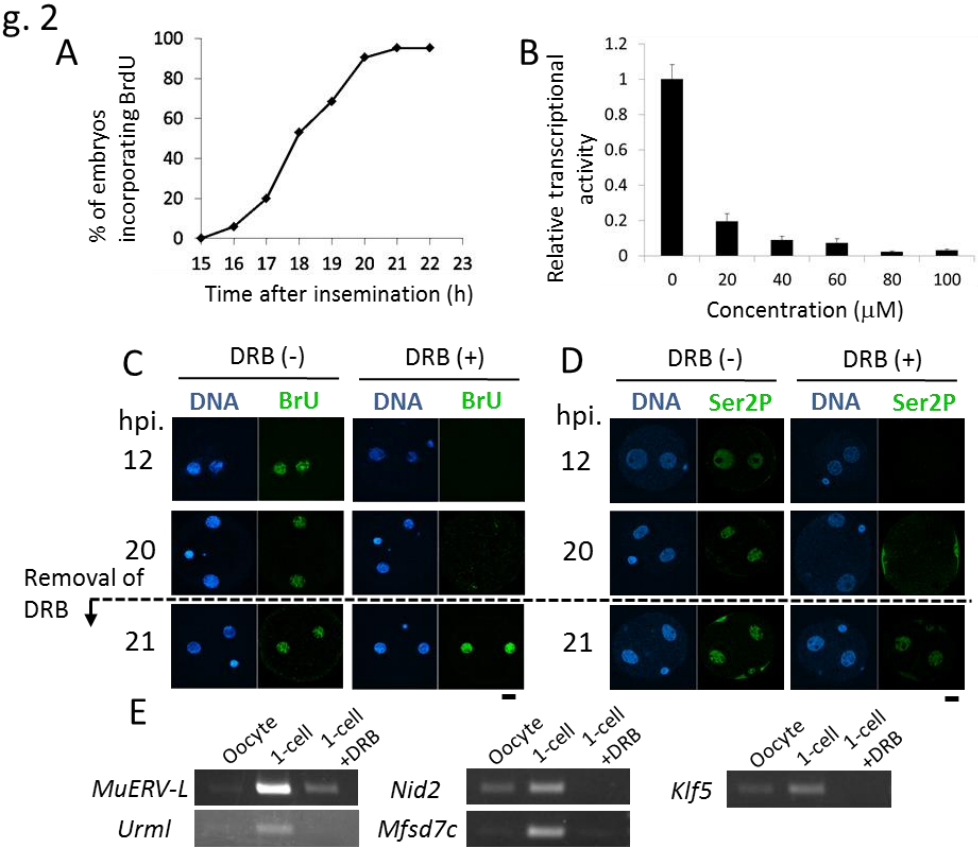

Fig. 2. Temporal inhibition of minor ZGA by 5, 6-dichlorobenzimidazole riboside (DRB).
(A) Determination of the timing of S phase at the 2-cell stage. The occurrence of DNA replication was determined by the incorporation of bromodeoxyuridine (BrdU). The incorporated BrdU was detected by immunocytochemistry with anti-BrdU antibody. The x axis represents the time after insemination. The y axis indicates the percentage of embryos in which BrdU was detected in their nuclei. More than 20 embryos were observed in each time point. (B) Determination of the minimum concentration of DRB to inhibit the transcription during minor ZGA. The embryos were treated with DRB between 4 and 12 h after insemination. The transcriptional activity was measured by the amount of incorporation of 5-bromouridine (BrU) at 12 h after insemination. The incorporated BrU was detected by immunocytochemistry with anti-BrdU antibody which recognizes BrU and quantified by the signal intensity of immunostain. The transcriptional activity in the embryos treated with dimethyl sulfoxide (0 μM), which was the solvent of DRB, was set as 1 and the relative activities were calculated for other samples. More than 20 pronuclei were analyzed in each sample. Polyspermic or parthenogenetic embryos were removed from analysis data. Error bars represent S.E.M. (C), (D) Validation of the inhibition and recovery of transcriptional activity (C) and phosphorylated RNA polymerase II (D) by the treatment and removal of DRB. The embryos were cultured with DRB between 4 and 20 h and then transferred into the DRB-free medium. The embryos were collected 12, 20 and 21 h after insemination for the determination of transcriptional activity and the phosphorylation of RNA polymerase II on the C-terminal Ser2 (Ser2P). The transcriptional activity was examined by the incorporation of BrU as described above. Phosphorylated Ser2 was detected by immunocytochemistry with anti-phosphorylated Ser2 antibody. More than 20 pronuclei and nuclei were examined in each sample. Polyspermic or parthenogenetic embryos were removed from analysis data. hpi indicates hours post insemination. Scale bar represents 20 μm. (E) The inhibitory effect of DRB on the expression of the genes that are transcribed during minor ZGA. The embryos were treated with DRB between 4 and 12 h after insemination. The expression of the genes was examined by reverse transcription and polymerase chain reaction. The experiments were performed three times and similar results were obtained.

Fig. 3

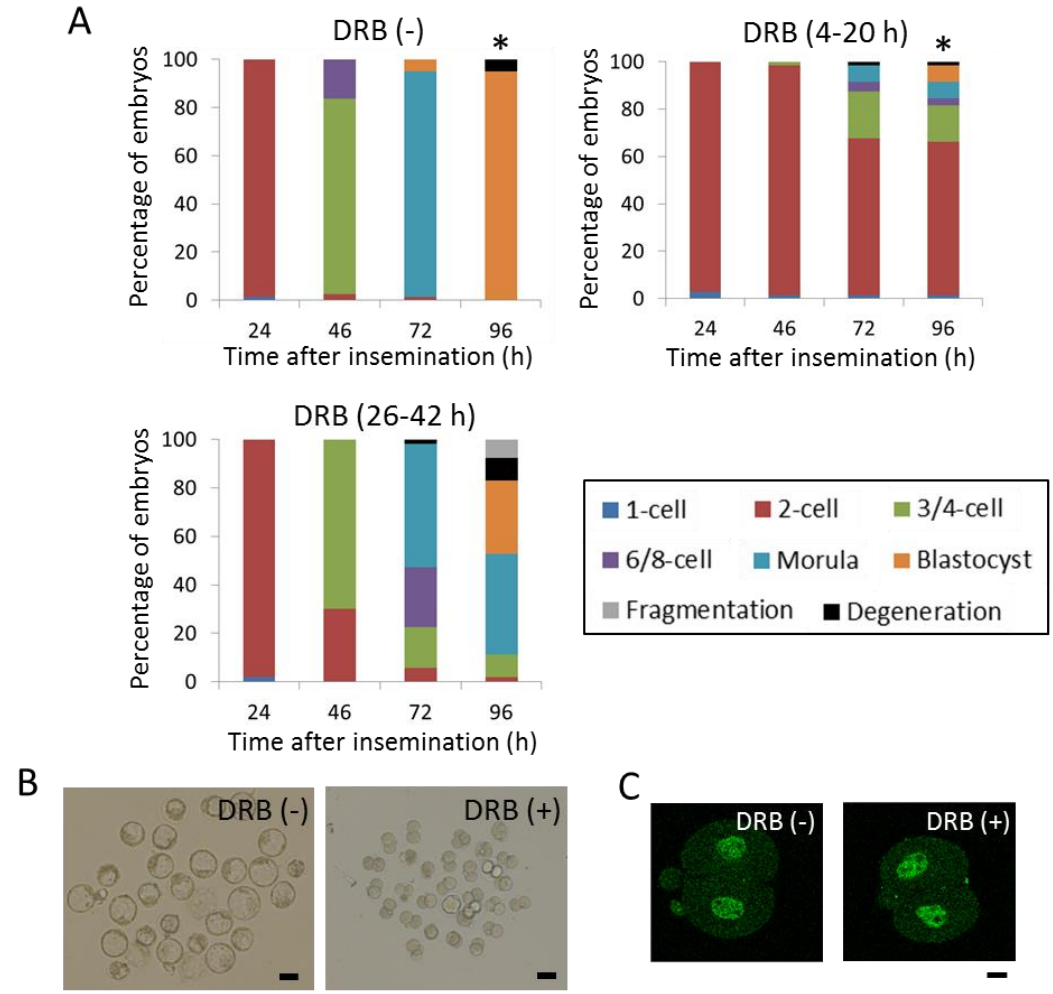

Fig. 3. Effect of the temporal inhibition of minor ZGA on the preimplantation development.
(A) Development of the embryos that were temporarily treated with 5,6-dichlorobenzimidazole riboside (DRB). DRB (-) indicates the embryos which were treated with dimethyl sulfoxide, the solvent of DRB, between 4 and 20 h after insemination. DRB (4-20 h) and DRB (26-42 h) represent the embryos which were treated with DRB between 4 and 20 h and between 26 and 42 h after insemination, respectively. The x axis indicates the time points of observation. The y axis shows the percentages of the embryos that developed to the indicated developmental stages. The experiments in which more than 30 embryos were observed were conducted three times, and the data are accumulated and analyzed by chi-square test. Asterisk represents significant differences of the percentage of embryos developed to blastocyst stage ($P < 0.05$). (B) The morphology of embryos treated with or without DRB at 96 h after insemination. DRB (-) and (+) represent the embryos treated with dimethyl sulfoxide and DRB, respectively, between 4 and 20 h after insemination. Scale bar indicates 100 µm. (C) The second round of DNA replication in the embryos which had been treated with DRB. The embryos were cultured with DRB between 4 and 20 h after insemination and then transferred to DRB-free medium. They were collected for the determination of DNA synthesis by the incorporation of bromodeoxyuridine (BrdU). Incorporated BrdU were detected by immunocytochemistry with anti-BrdU antibody. The experiments were conducted three times and more than 10 embryos were analyzed in each experiment. The incorporation of BrdU was detected in all (more than 97% of) embryos analyzed. Scale bar indicates 20 µm.

Fig. 4

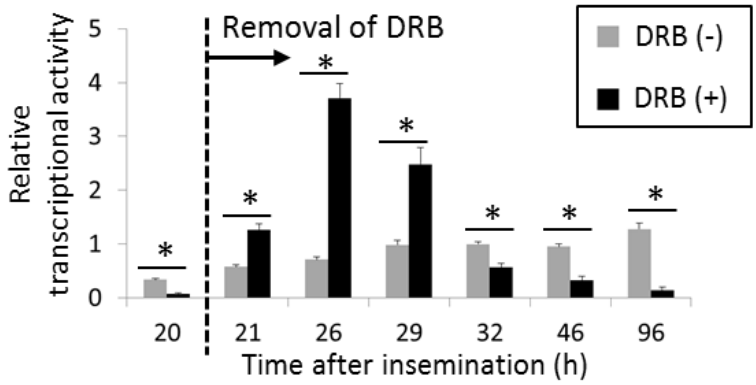

Fig. 4. Effect of the temporal inhibition of minor ZGA by 5,6-dichlorobenzimidazole riboside (DRB) on the transcriptional activity after its removal.
The embryos were cultured with DRB between 4 and 20 h after insemination and then transferred to DRB-free medium. The transcriptional activity was measured by the amount of 5-bromouridine (BrU) incorporation at the indicated time points. The incorporated BrU was detected by immunocytochemistry with anti-BrdU antibody and quantified by the signal intensity of immunocytochemistry. The transcriptional activity in the embryos which had not been treated with DRB (DRB (-)) at 32 h after insemination was set as 1. The experiments in which more than 20 nuclei were analyzed were conducted three times. The column and error bar represents mean ± S.E.M. Asterisks represent significant differences (by student's *t*-test; $P < 0.05$).

Fig. 5

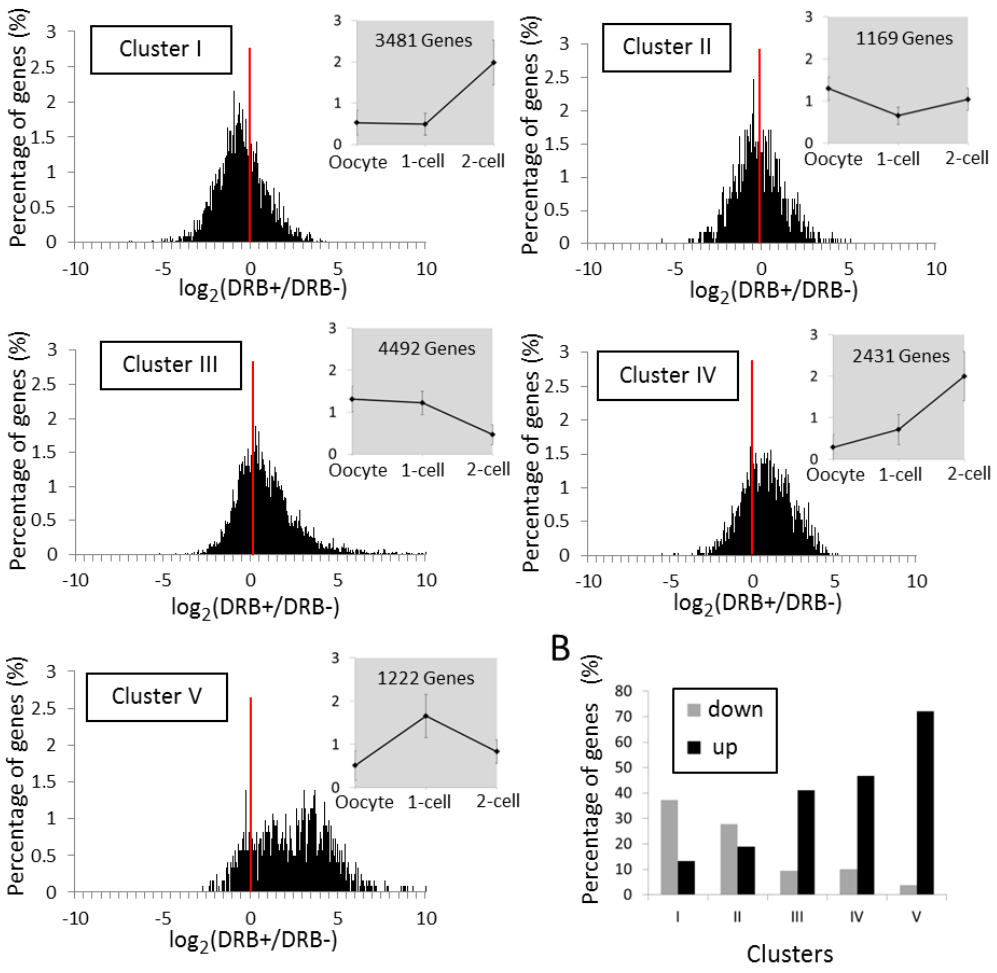

Fig. 5 Effect of the temporal inhibition of minor ZGA by 5,6-dichlorobenzimidazole riboside (DRB) on the gene expression at the late 2-cell stage.

The expression of genes was measured by RNA sequencing 12 h after removal of DRB (32 h after insemination). (A) Classification of genes by k-mean nonhierarchical clustering analysis based on their expression patterns in the MII stage oocytes, 1-cell (13 h after insemination) and 2-cell stage embryos (32 h after insemination)(Line graphs with grey backgrounds). The genes that were mapped by less than 11 reads were omitted from the analysis. The y axis represents the relative expression level. The average expression levels in the oocytes, and 1- and 2-cell stage embryos were set as 1. Error bars represent S.D. The histograms with white backgrounds show the distribution of the up- and down-regulated genes by the treatment with DRB in each cluster. The x axis represents the ratio of their expression levels between the embryos treated with DRB (DRB+) and those of control ones which had not been treated with the drug (DRB-). The y axis represents the percentage of genes with particular DRB+/DRB- ratio in total number belonging to each cluster. The red vertical line represents the position of 0. (B) The number of genes highly up- or down-regulated by the temporal inhibition of minor ZGA in each cluster. The genes, in which their expression levels were more than 2 times higher and less than a half, respectively, in the DRB-treated embryos than DRB-untreated ones, were defined as highly up- and down-regulated, respectively.

Fig. 6

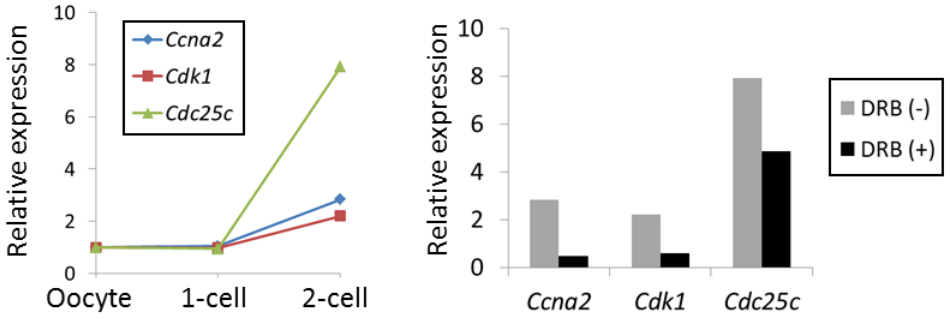

Fig. 6. Repression of the expression of mitosis-regulating genes by the temporal inhibition of minor ZGA.
The expression levels of Ccna2, Cdk1 and Cdc25c were obtained from the RNA sequencing data. The expression level of MII stage oocytes was set as 1. The left panel shows the relative expression levels in oocytes, 1- and 2-cell stage embryos which had not been treated with 5,6-dichlorobenzimidazole riboside (DRB). The right panel shows the relative expression levels in the 2-cell stage embryos which had been treated with and without DRB (DRB+ and DRB-, respectively).

Chapter II

The exploration of functional factors and the elucidation of transcriptional regulatory mechanism during minor ZGA

Result

Pervasive transcription from intergenic regions during minor ZGA

It has been reported that the transcripts are not spliced at the 1-cell stage (Yamamoto et al., 2012), indicating that functional proteins are not synthesized from these transcripts. Therefore, I focused on the transcripts from intergenic regions, since recent emerging evidence revealed that long intergenic non-coding RNA (lincRNA; Guttman et al., 2009) and small RNAs, *e.g.* siRNA, miRNA and piRNA, transcribed from non-coding regions have specific functions on various biological processes (Cieśla et al., 2014; Dogini et al., 2014).

First, I analyzed the genome wide expression from intergenic regions. When I overviewed the transcribed regions on the whole genome, I found that a pervasive transcription occurs in the intergenic regions of 1-cell stage embryos (Fig. 7A). To confirm it quantitatively, I divided the intergenic regions across the entire genome into 1-kb sequences and enumerated divided regions in which at least a single read was mapped uniquely. The number of mapped regions was threefold higher in 1-cell stage embryos than oocytes (Fig. 7B). In the embryos at the late 2-cell stage, the number of mapped regions was smaller than that of 1-cell stage embryos and it decreased gradually with development and reached levels similar to those of oocytes at the morula stage. When 1-cell stage embryos were treated with DRB, the number of mapped regions remained similar to that in oocytes (Fig. 7C). These results suggested that pervasive transcription occurs in the intergenic regions during minor ZGA, but that this was repressed after the occurrence of major ZGA.

Involvement of intergenic RNAs transcribed during minor ZGA in the preimplantation development

Since it was reported that some lincRNAs function without being spliced (Hutchinson et al., 2007), it was possible that a lincRNA(s) was involved in the preimplantation development. To identify it (them), I investigated the intergenic RNAs which are specifically expressed at the 1-cell stage. Since previous study showed that lincRNA contains evolutionary conserved sequences which are predicted as functional domains (Guttman et al., 2009), I sought for the intergenic RNAs which contained evolutionary conserved sequences and were specifically expressed in 1-cell stage embryos. I first selected the intergnic regions mapped by one or more reads at the 1-cell stage but not other embryonic stages using RNA sequence data, and then examined whether or not those regions contain the sequences conserved among humans, mice, dogs and horses by using multiple alignments data of UCSC genome browser (Fig. 8A). By this primary selection, I obtained 25 candidates for lincRNA specifically expressed at the 1-cell stage. To further confirm the specific expression of these candidates at the 1-cell stage, I examined their expression in oocytes, preimplantation embryos and adult tissues by RT-PCR. The results showed that 4 species of intergenic RNAs were specifically expressed at the 1-cell stage (Fig. 8B). Intriguingly, these candidates for lincRNA were not registered in RNA db, the data base for non-coding RNA. To assess which strands are transcribed for these candidates, I conducted RT-PCR in which reverse transcription was performed using primers specifically binding to plus or minus strand in intergenic RNAs coding regions. The candidates, linc-1, 2, 3 and 4 were transcribed from plus, minus, plus and minus strands, respectively (Fig. 8C). Since the candidate RNAs might contain an open reading frame that is translated into a functional protein, I evaluated the

protein coding potential in the above-mentioned conserved sequences in the candidates, using Coding Potential Calculator (Kong et al., 2007). The result indicated that all of 4 candidates had a high potential for non-coding. Thus, it was very likely that the intergenic RNA that I had identified were novel lincRNAs specifically expressed at the 1-cell stage.

To assess whether 1-cell specific intergenic RNAs are required for the preimplantation development, their expression was reduced by LNA-DNA gapmers which had been reported to successfully reduce major satellite transcripts in preimplantation embryos (Probst et al., 2010). I injected into 1-cell stage embryos with two different sequences for each of 4 intergenic RNAs (linc-1, 2, 3 and 4). All of these gampers, except for the second one (gapmer B) targeting linc-4, successfully reduced the expression of their target RNAs (Fig. 9A). When non-targeting control gapmers or gapmers targeting a lincRNA which was not expressed at the 1-cell stage was injected, more than 80% of embryos developed to the blastocyst stages (Fig. 9B). However, the reduction of development was observed in the embryos which had been injected with the gapmers targeting any of linc-1-4. Only 30% or less embryos developed to the blastocyst stage (Fig. 9B). These results suggested that 1-cell specific intergenic RNAs were essential for embryonic development.

Regulation of pervasive transcription from intergenic regions

It is likely that pervasive transcription from intergenic regions is associated with the transcriptionally permissive state before the second round of DNA replication. Previous studies using reporter gene assays demonstrated that 1-cell stage embryos are in a transcriptionally permissive state, in which enhancers are not required for gene

expression, and that a transcriptionally repressive state requiring enhancers is established during DNA replication at the 2-cell stage (Nothias et al., 1995). When the DNA replication was inhibited, the enhancer independent transcription still occurred in 2-cell stage embryos (Wiekowski et al., 1993; Henery et al., 1995). This transcriptionally permissive state allowed for promiscuous expression without an enhancer from the intergenic regions during minor ZGA; establishment of the repressive state suppressed promiscuous expression during major ZGA. To address this hypothesis, we inhibited DNA replication in 2-cell stage embryos by treatment with aphidicolin, an inhibitor of DNA polymerase, and analyzed the RNA sequencing data and transcription from intergenic regions. We found that the pervasive transcription from intergenic regions still occurred in the embryos at the late 2-cell stage in which DNA replication was inhibited; the number of transcribed intergenic regions was higher in 2-cell stage embryos treated with aphidicolin than in 1-cell stage embryos (Fig. 10A). To confirm the association of the permissive state with intergenic transcription during minor ZGA, we selected intergenic regions to which a number of reads were uniquely mapped in 1-cell stage embryos but not oocytes, and examined their expression by RT-PCR. We first confirmed that transcription of these two intergenic regions occurred only in 1-cell stage embryos but not in oocytes or 1-cell stage embryos treated with DRB (Fig. 10B). Their expression levels decreased at the 2-cell stage but remained high upon inhibition of DNA replication by aphidicolin (Fig. 10C).

Taken together, these results suggested that a transcriptionally permissive state allows for promiscuous expression from the intergenic regions during minor ZGA, and that establishment of the repressive state, which is coupled to DNA replication, suppresses promiscuous expression.

Core-promoter-element-independent pervasive transcription in minor ZGA

Since transcription requires core promoter elements in eukaryote cells, I analyzed which elements are involved in pervasive transcription from intergenic regions. I examined the presence of DPE, Initiator (Inr), CCAAT, TATA box, BRE and GC box in 500 bp upstream and 100 bp downstream of transcribed intergenic regions. Although DPE, Inr and CCAAT were present at a high rate, a similar result was obtained in randomly selected 600 bp regions from untranscribed intrgenic regions (Fig. 11A), suggesting that pervasive intergenic transcription is not caused by a particular core promoter element.

In mammalian somatic cells, the promoter regions of many genes have a high G/C content (Sandelin et al., 2007; Fenouil et al., 2012). In addition, previous reporter gene experiments using a vector without any insertion of promoter sequences showed that transcription occurred independently of known core promoter elements but is associated with high G/C content regions in 1-cell stage embryos (Cao et al., 2013). Therefore, to investigate whether or not the sequences with high G/C content are associated with the pervasive transcription from the intergenic regions, I counted the number of transcribed regions around the G/C-rich regions using RNA sequence data. The number of transcribed 1 kb upstream and downstream of G/C-rich (more than 60%) regions were the largest in the 1-cell stage embryos (Fig. 11B), suggesting that transcription occurs around G/C-rich regions at the higher rate in 1-cell stage embryos. Thus, G/C-rich regions seem to be associated with the pervasive transcription observed in 1-cell stage embryos.

Discussion

In this study, I found that pervasive transcription occurs in intergenic regions. Thus, many functional non-coding RNAs are likely produced during minor ZGA. A recent study demonstrated that newly synthesized mRNAs are hardly translated into functional proteins at the 1-cell stage because of the deficiency of splicing machinery (Yamamoto et al., 2012). Recent emerging evidence revealed that non-coding RNAs play key roles in various biological processes and that some non-coding RNAs function without undergoing splicing, *e.g.* siRNA, miRNA, piRNA and some lincRNAs (Hutchinson et al., 2007; Cieśla et al., 2014; Dogini et al., 2014). It was reported that some of these non-coding RNAs are involved in the regulation of gene expression and that siRNA has important functions for preimplantation development (Tam et al., 2008). In addition, I found that intergenic RNAs which are transcribed specifically at the 1-cell stage and the candidates for novel lincRNAs were essential for preimplantation development (Fig. 9B). Intriguingly, the present study demonstrated that minor ZGA is indispensable for the activation of subsequent major ZGA (Chapter I). Therefore, the intergenic RNAs identified in my study may play a key role in the alteration of the regulatory mechanism for gene expression from minor ZGA into major one. It has been reported that some lincRNAs involve transcription through epigenetic modifications and the interaction with transcription factors (Zhao et al., 2010; Bergmann and Spector, 2014; Wang et al., 2014). Intergenic RNAs transcribed during minor ZGA may contribute to activation of the transcription factors or change the epigenetic status for occurrence of major ZGA. In addition, it was reported that *HOTAIR*, a lincRNA, was associated with the degradation of Ataxin-1 and Snurportin-1 through an ubiquitin-proteasome pathway (Yoon et al.,

2013). Thus, novel intergenic RNAs may be involved in the rapid degradation of the proteins that are involved in the oocyte-specific phenomenon, *e.g.* meiosis, but make detrimental effects on the preimplantation development after fertilization.

I have shown that all of four intergenic RNAs which I had identified were essential for preimplantation development (Fig. 9B). I prepared two types of LNA-DNA gampers with different sequences for each of these four intergenic RNAs, and found that the injection of any of these gampers caused the reduction of preimplantation development. However, in the embryos injected with the gapmers against linc-3 and -4, the patterns of the reduction were inconsistent between the two types of gapmers. In the experiments for the knock-down of linc-4, the embryos injected with gapmer 4A showed the reduction of development at the 4-cell stage. However, those with gapmer 4B did not show any reduction at this stage, and they develop to the morula stage but not the blastocyst one (Fig. 9B). This different phenotypes between gapmer 4A and B seems to be caused by the efficiency of reduction of target RNA. The injection of gapmer 4A successfully reduced the expression of linc-4, whereas gapmer 4B caused the moderate reduction (Fig. 9A). In the experiments in which linc-3 was targeted, although the efficiency of reduction was at the same level between the embryos injected with gapmer 3A and 3B (Fig. 9A), the reduction of development was severer in the embryos injected with gapmer 3B: the embryos injected with gapmer 3B showed the reduction at the 4-cell stage, whereas those with gapmer 3A developed to the morula stage (Fig. 9B). After the hybridization of LNA-DNA gapmers with their target RNA, they are sliced by RNase H (Braasch and Corey, 2001). The sliced RNA that bound to gapmer A might still have the function which supported the development to the morula stage.

The pervasive transcription is likely caused by the loosened chromatin structure

during minor ZGA. Generally, chromatin structure is repressive for transcription. Enhancers are required by transcription factors to be accessible to promoters in repressive chromatin. However, previous studies suggested that transcription is regulated independently of enhancers at the 1-cell stage (Wiekowski et al., 1991; Majumder et al., 1993; Schultz, 1993). It is known that the chromatin is decondensed and heterochromatin domains are present only in the peripheral regions of nucleolei in the embryos at the 1-cell and early 2-cell stages (Akiyama et al., 2011). A previous study showed that the genome DNA was more sensitive to DNase I at the 1- than the late 2-cell stage, suggesting that the chromatin is loosened in the 1-cell stage embryos (Cho et al., 2002). Therefore, this loosened chromatin structure seems to enable transcription factors to access to the promoters without the involvement of enhancers. In the present study, I have shown that various core promoter elements are present on the upstream of intergenic regions transcribed at the 1-cell stage, but that these elements are also found in the randomly-selected regions (Fig. 11A), which would deny the hypothesis that a core promoter element is responsible for the pervasive intergenic transcription. Since the areas around G/C-rich regions were transcribed at a high rate in the 1-cell stage embryos (Fig. 11B), these regions may be involved in the regulation of pervasive transcription.

Fig. 7

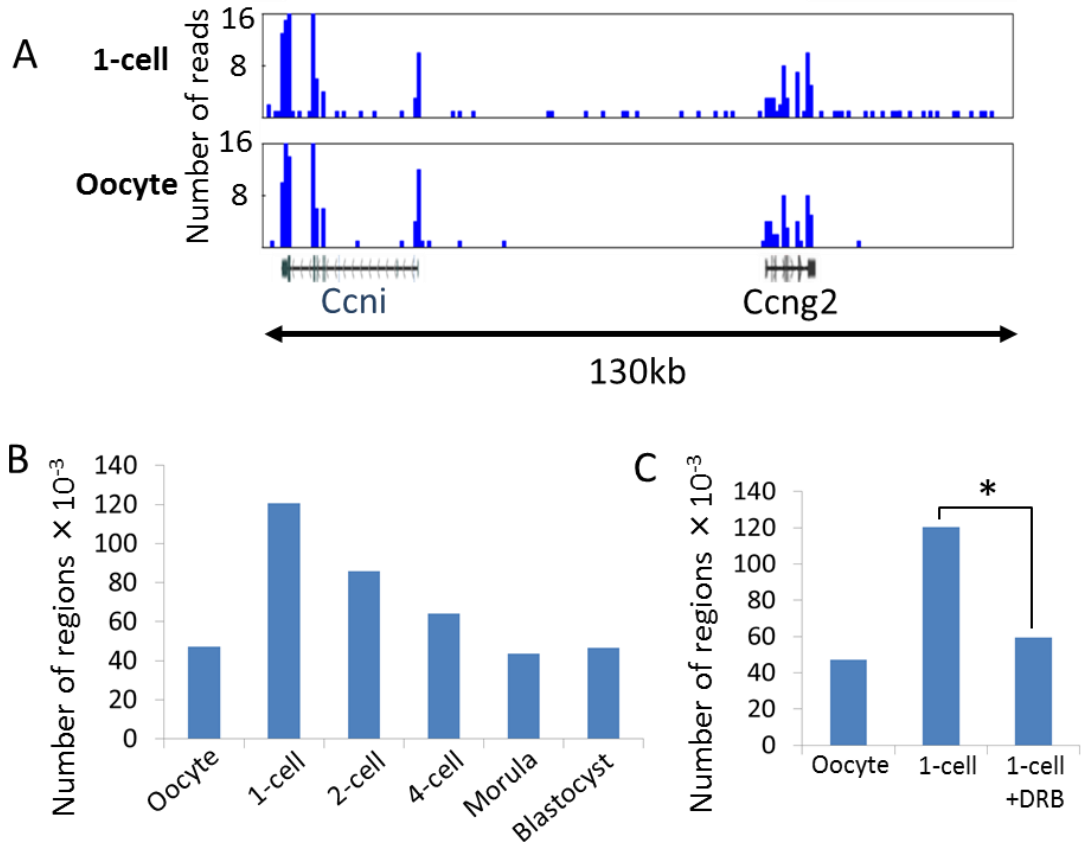

Figure 7. Pervasive transcription from intergenic regions in 1-cell stage embryos.
(A) Overview of transcribed regions in the genome. The reads obtained in RNA sequences for 1-cell stage embryos (1-cell) and oocytes were mapped to the region containing *Ccni* and *Ccng2* genes on chromosome 5. Blue bars represent the numbers of mapped reads. (B) Transcription from intergenic regions in oocytes and pre-implantation embryos. Intergenic regions were divided into 1-kb segments across the whole genome, and the number of divided regions to which at least a single read was uniquely mapped was determined. (C) Effect of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) on intergenic transcription in 1-cell stage embryos. The number of transcribed intergenic regions in DRB-treated 1-cell stage embryos was determined as described in (B). An asterisk indicates a significant difference by chi-square test ($P < 0.05$).

Fig. 8

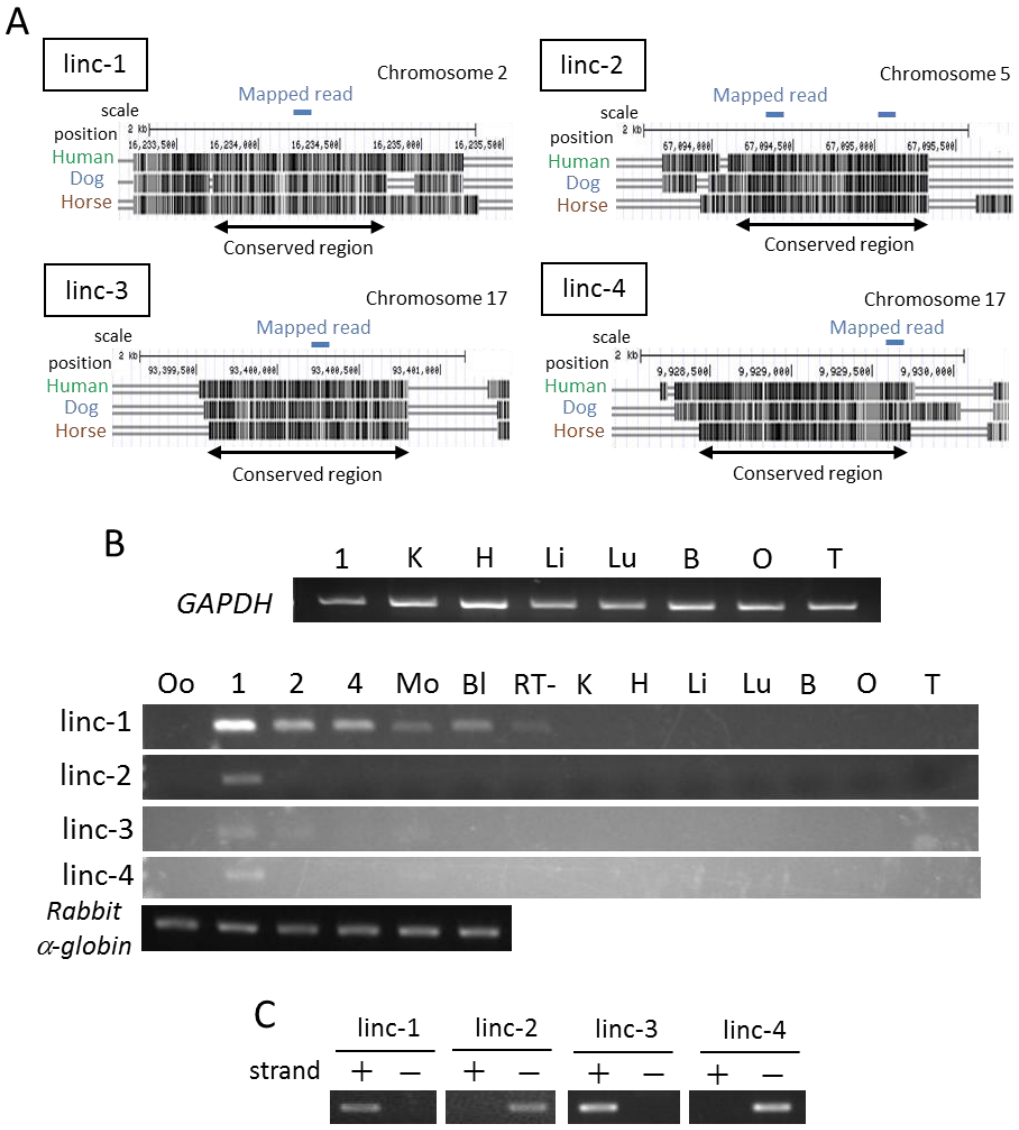

Figure 8. Identification of intergenic RNAs specifically expressed in 1-cell stage embryos.
(A) Intergenic regions conserved in mice, human, dogs, and horses were searched and visualized in mouse genome using UCSC genome browser of multiz Alignments. The black and grey lines represent the homologous regions between 4 mamalian species. (B) RT-PCR to assess the expression of 1-cell specific intergenic RNA (linc-1, -2, -3, and -4) in oocytes (Oo), 1-cell stage embryos (1), 2-cell stage embryos (2), 4-cell-stage embryos (4), morula-stage embryos (Mo), blastocyst-stage embryos (Bl), kidney (K), heart (H), liver (Li), lung (Lu), brain (B), testis (T) and ovary (O). RT- represents cDNA sample which was not reverse transcribed in 1-cell stage embryos. Rabbit *α-globin* was an external control to monitor the efficiency of total RNA isolation from the oocytes and embryos. The amount of total cDNAs in each tissue and 1-cell stage embryos were adjusted to a similar level based on the expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). (C) Determination of transcribed strand in intergenic RNAs by reverse transcription using primers specifically binding to plus or minus strand in intergenic RNAs coding genomic regions, which was followed by PCR.

Fig. 9

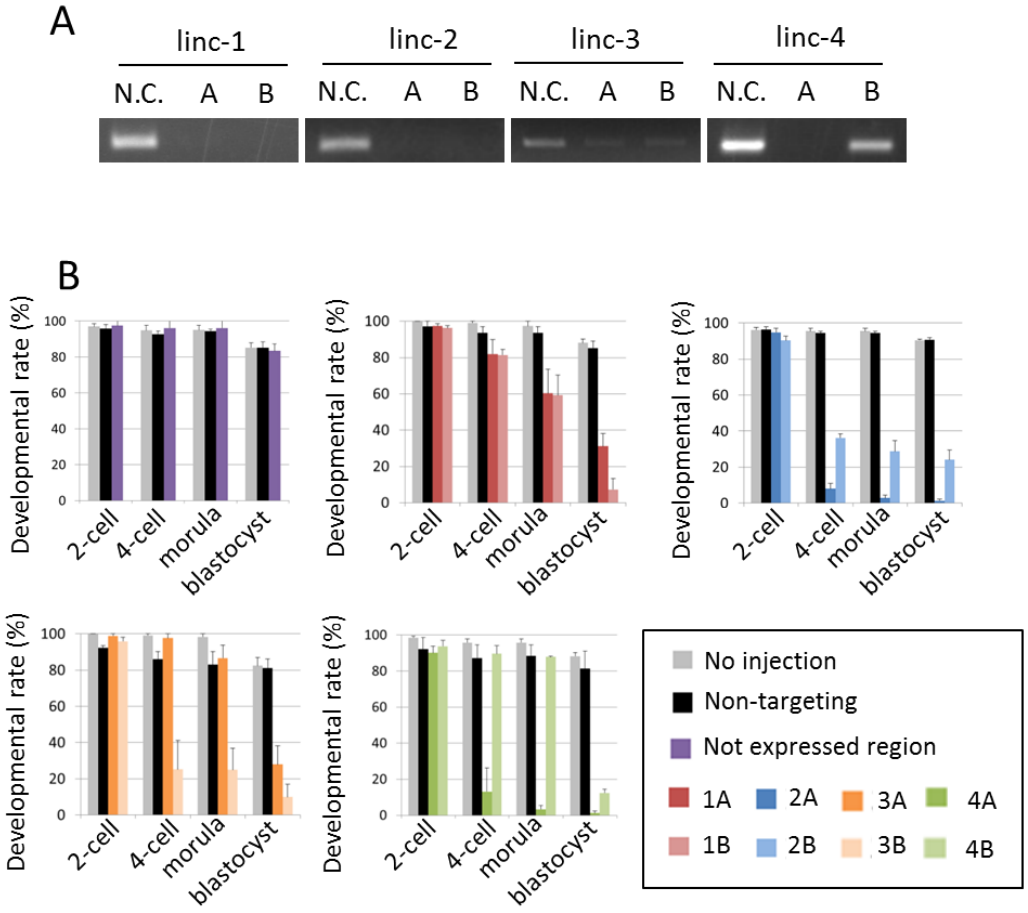

Figure 9. Function of 1-cell specific intergenic RNAs for preimplantation development.
(A) Assessment for the depletion of 1-cell specific intergenic RNAs by RT-PCR in the 1-cell stage embryos injected with LNA-DNA gapmers. The gapmer A and B specifically bind to the different positions in the corresponding target RNA. N.C represents non-targeting negative control LNA-DNA gapmer. (B) Development of the embryos injected with LNA-DNA gapmers. Gray column represents the embryos which did not undergo the injection. A color legend, "Non-targeting" (Black column) and "Not expressed region" (purple column), represent the embryos which were injected with a negative control gapmer whose sequence is not present in mouse genome and a gapmer targeting the intergenic region which was not transcribed at the 1-cell stage, respectively. The symbols, 1A and B, 2A and B, 3A and B, and 4A and B represent gapmers specifically targeting linc-1 (red), -2 (blue), -3 (yellow), and -4 (green), respectively. The experiments in which more than 20 embryos were observed were conducted three times. The error bar represents mean ± S.E.M.

Fig. 10

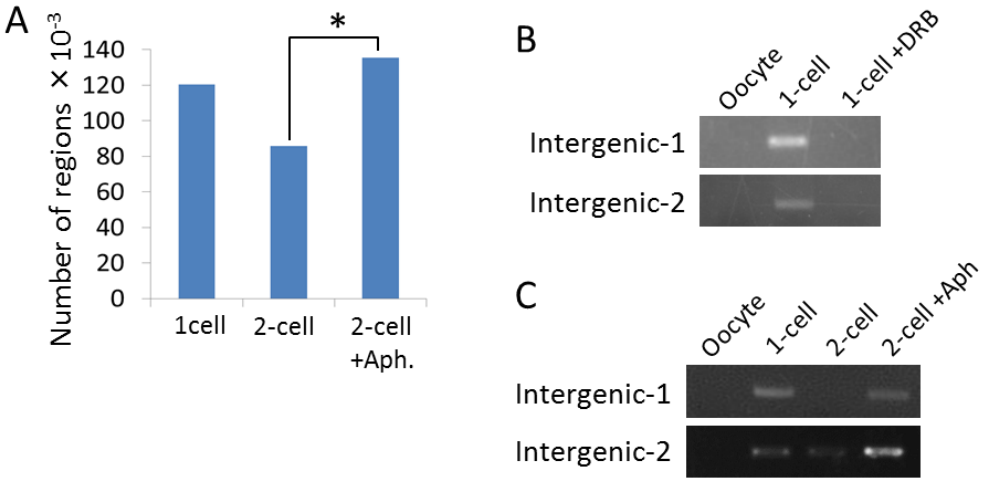

Figure 10. Role of the second round of DNA replication in the repression of pervasive intergenic transcription at the 2-cell stage.

(A) Effect of aphidicolin (Aph) on intergenic transcription in 2-cell stage embryos. The number of intergenic regions transcribed in 2-cell stage embryos, in which the second round of DNA replication was inhibited by the treatment with aphidicolin, was determined as described in Figure 7B. An asterisk indicates a significant difference by chi-square test ($P < 0.05$). (B) Intergenic transcription in oocytes and 1-cell stage embryos treated with and without 5,6-dichlorobenzimidazole riboside (DRB) were examined by RT-PCR. Intergenic-1 and -2 represented RT-PCR products generated from the regions of 171636150–171636386 and 16216910–16217675, respectively, on chromosome 2. (C) The effect of inhibiting the second round of DNA replication in intergenic transcription at the 2-cell stage was examined by RT-PCR. 2-cell stage embryos were treated with aphidicolin (Aph) 15 h after insemination to inhibit the second round of DNA replication. The experiments were conducted in triplicate and similar results were obtained.

Fig. 11

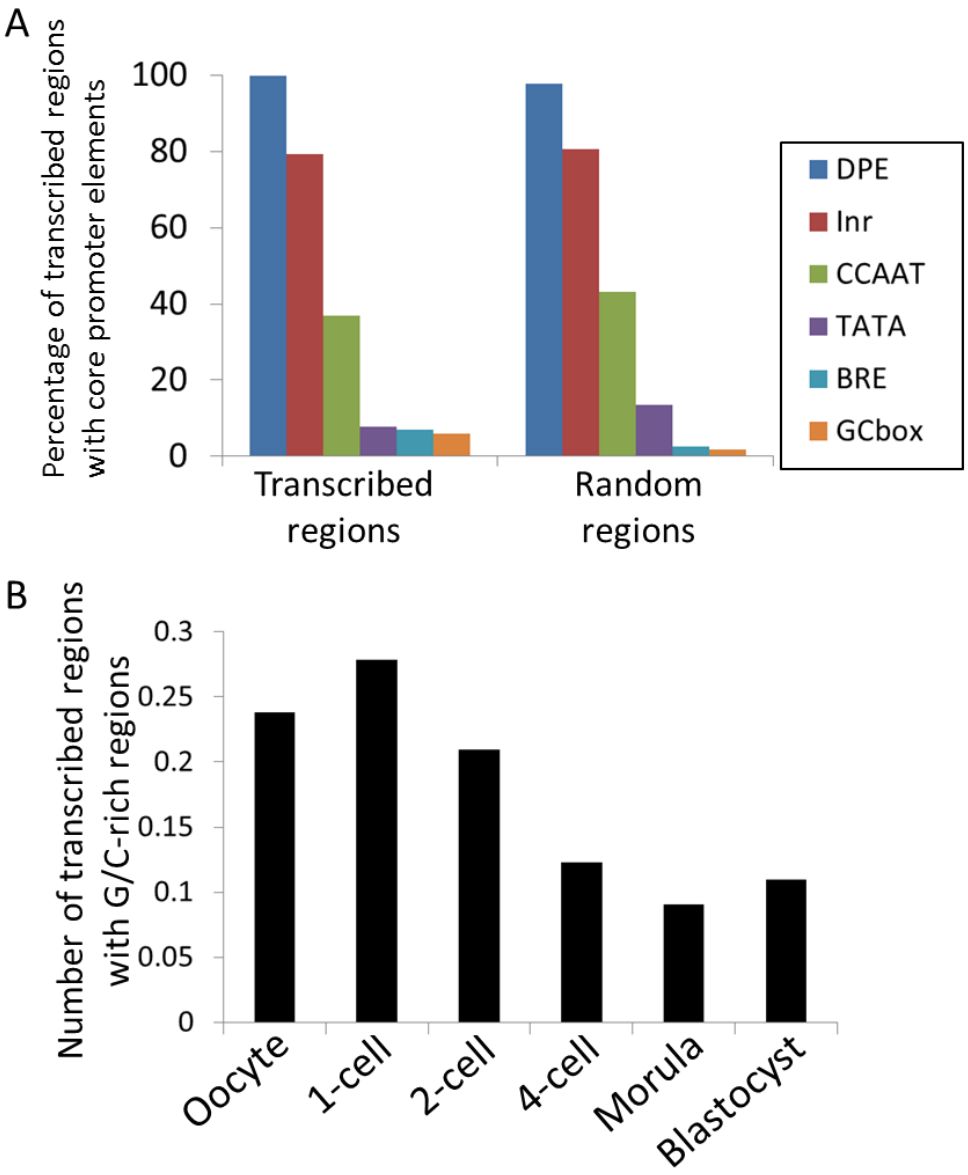

Figure 11. Association of high G/C regions with pervasive transcription.
(A) The presence of core promoter elements was examined in 1-cell stage embryos in the 500 bp up- and 100 bp down-stream of the end of transcribed intergenic regions. When two or more reads of RNA sequencing were mapped within 1-kb, these reads were regarded to be derived from a single transcribed intergenic region. The mapped reads in both of oocytes and 1-cell stage embryos and less than 100 bp of length and total 5 reads of transcribed intergenic regions were removed from the further analysis. Random regions represent the randomly selected 30,000 of 600 bp regions from intergenic regions that were not mapped by any reads in 1-cell stage embryos. (B) The assessment of the involvement of high G/C regions in pervasive transcription. The number of transcribed 1-kb up- and downstream of G/C-rich regions which were mapped by at least single read was counted. Since the number of transcribed up- or downstream regions increases according to the number of mapped regions as shown in Fig. 7B, the total number of transcribed up- and downstream regions was normalized by the number of mapped regions in each stage.

General discussion

In this thesis, I have shown two important findings about minor ZGA. The first one is the significance of minor ZGA in the regulation of development. It has been known that minor ZGA occurs in various species. However, the biological significance and mechanism of this gene expression has not been clarified, because it has been thought that minor ZGA is dispensable for the regulation of development. The embryos in which minor ZGA were inhibited by α-amanitin, an irreversible Pol II inhibitor, cleaved into 2-cell stage followed by DNA replication and then arrested at G2 phase (Warner and Versteegh, 1974), which led to the idea that major ZGA but not minor ZGA is essential for the regulation of preimplantation development. However, in my study, I inhibited minor ZGA temporarily by using DRB, a reversible inhibitor, and obtained the first evidence that minor ZGA is indispensable for embryonic development through the activation of subsequent major ZGA.

The second important finding in this thesis is that intergenic regions are pervasively transcribed during minor ZGA and that several species of RNAs transcribed from those regions are involved in the regulation of preimplantation development. Previous analyses using microarray (Hamatani et al., 2004; Wang et al., 2004) or RNA sequencing (Park et al., 2013; Xue et al., 2013) have not provided any information about this unique transcription from intergenic regions during minor ZGA, because the probes of micro arrays in the previous analyses targeted only known gene regions and the recent RNA sequencing analysis focused on only known protein coding or annotated non-coding regions but not global intergenic regions. However, the present analysis of RNA sequencing explored unknown intergenic regions. Thus, my study is the first

report which has shown the unique mechanism of transcriptional regulation in intergenic regions during minor ZGA.

Several studies suggested that the chromatin structure at the 1-cell stage is looser than that at other embryonic stages (Cho et al., 2002; Ahmed et al., 2010). Although the loosened chromatin structure seems to induce pervasive transcription during minor ZGA, the mechanism of decondensation in the chromatin of 1-cell stage embryos is unknown. It is known that the chromatin structure is regulated by chromatin remodeling factors, epigenetic modifications and histone variants. Some of these factors are known as the chromatin regulator in 1-cell stage embryos. The artificially increased level of HMG-I protein, which is a chromatin remodeling factor, by the injection with the recombinant HMG-I induced chromatin decondensation and accelerated the timing of minor ZGA (Beaujean et al., 2000). In addition, the elevated level of histone acetylation by tricostatin A, which is an inhibitor for histone deacetylases, induced to reorganize the chromatin structure from that of somatic cells into that of 1-cell stage embryos in transferred embryos with somatic nucleus (Martin et al., 2006). This report suggests that histone acetylases also promoted the decondensation of chromatin in 1-cell stage embryos. Thus, HMG-I and histone acetylases possibly induce chromatin decondensation during minor ZGA phase. The unique component of histone variants in the pronucleus at the 1-cell stage is likely also involved in the loosened chromatin structure. Although there are several H2A variants, only H2A.X is abundantly incorporated into the pronuclei of 1-cell stage embryos (Nashun et al., 2010). The H3 variants also showed a unique pattern of the deposition in the nucleosome of 1-cell stage embryos: only H3.3 was incorporated in the pronuclei of 1-cell stage embryos (Akiyama et al., 2011). Therefore, limited species of histone variants in the nucleosome

of 1-cell stage embryos may also contribute to the loosen state of chromatin during the phase of minor ZGA.

Minor ZGA has been interested by few people and its regulatory mechanism has not been studied well, since it has been thought to have no function in development. However, in this thesis, I have shown that minor ZGA is indispensable and regulated by a unique mechanism. These new findings contribute to understand the mechanism regulating the development and various cellular functions, because minor ZGA is the initiation of gene expression program regulating the entire process of development and cellular events.

Materials and methods

Collection and culture of oocytes, embryos and adult tissues

Metaphase-II-stage oocytes (oocytes) were obtained from superovulating 3-week-old C57BL6/J or BDF1 female mice (SLC Japan, Shizuoka) treated with 5 IU of pregnant mare serum gonadotropin (PMSG; ASKA Pharmaceutical Co., Tokyo) followed 48 h later by 5 IU of human chorionic gonadotropin (hCG; ASKA Pharmaceutical). Oocytes were collected from the ampullae of oviducts 15 h post-hCG injection and transferred to human tubal fluid medium (HTF) (Lawitts and Biggers, 1993) supplemented with 10 mg/ml BSA (Sigma-Aldrich, Saint Louis, MO). For the oocytes of C57BL6/J female mice, the spermatozoa were collected from the caudal epididymides of adult C57BL6/J male mice (SLC Japan). When the oocytes of BDF1 female mice were inseminated, the spermatozoa were obtained from the caudal epididymides of adult ICR male mice (SLC Japan). The spermatozoa were pre-incubated for 2 h in TYH medium (Toyoda et al., 1971) or HTF medium supplemented with 10 mg/ml BSA for the oocytes of C57BL6/J female mice or BDF1 female mice, respectively, in an atmosphere of 5% CO_2/95% air at 38°C. The collected oocytes were inseminated with pre-incubated spermatozoa in HTF supplemented with 10 mg/ml BSA. Between four and six hours after insemination, the fertilized oocytes were washed and cultured in potassium simplex optimized medium (Quinn and Begley, 1984). The 1-cell, 2-cell, 4-cell, morula and blastocyst, stage embryos of C57BL6/J mice were collected at 13, 32, 48, 72, and 96 h after insemination, respectively. Adult tissues (kidney, heart, liver, lung, brain, and ovary) were obtained from 8-week-old BDF1 female mice. Testis was collected from 8-week-old BDF1 male mice. The oocytes of BDF1 mice and the embryos developed

from the inseminated oocytes of BDF1 mice with the spermatozoa of ICR mice were subjected to most experiments, exceptionally for the preparation of RNA sequencing libraries.

Treatment with DRB and aphidicolin

1-cell stage embryos were transferred into KSOM containing 20, 40, 60, 80, 100, and 120 μM DRB (Sigma-Aldrich) at 4 h after insemination. Transcription was not initiated at 4 h after insemination (Aoki et al., 1997). When the embryos were temporary treated with DRB, the treated embryos were moved into KSOM without DRB at 20 or 42 h after insemination. This reason in detail was described in chapter I. 1-cell stage embryos of C57BL/6 mice were transferred into KSOM with 3 mg/ml aphidicolin (Sigma-Aldrich) at 16 h after insemination. The majority of the C57BL/6 mice embryos entered M phase at 16 h after insemination.

***In vitro* transcription assay**

BrU was incorporated into nucleuses using *in vitro* transcription assay as described previously (Aoki et al., 1997). Plasma membrane of embryos was permeabilized with 0.08% of Triton X-100 (Sigma-Aldrich) in a physiological buffer for 1 min at room temperature. After the embryos were briefly washed with a physiological buffer at three times, the embryos were incubated in a physiological buffer containing nucleotides and 0.4mM BrUTP (Sigma) for 15 min at 33 ºC. The transcription reaction was terminated by the permeabilization of nuclear membrane in a physiological buffer containing 0.2% of TritonX-100 for 3 min at room temperature. The permeabilized embryos were fixed in a physiological buffer containing 3.7% of paraformaldehyde (Wako, Osaka, Japan)

for 1 hour at room temperature. The fixed embryos subjected to Immunocytochemistry using anti-BrdU antibody (1:50 dilution; Roche Diagonostic, Indianapolis, IN, USA), which has the cross reactivity against BrU, and Alexa flour 488-conjugated anti- mouse IgG secondary antibody (1:100 dilution; Sigma).

Incorporation of BrdU

The embryos were transferred into KSOM medium containing 10 μM BrdU (Roche Diagonostic) at 14 h after insemination. The majority of BDF1 mice embryos were G2/M or M phase of 1-cell stage at 14 h after insemination. The embryos which incorporated BrdU were fixed in phosphate buffered saline (PBS) containing 3.7% of paraformaldehyde for 1 hour at room temperature. After washing with PBS containing 1% bovine serum albumin (BSA), the fixed embryos were incubated in 2 M HCl for 1 hour at 37 ºC. The embryos were washed in PBS containing 1% BSA and then neutralized in 0.1 M Tris-HCl (pH 8.5) for 15 min at room temperature. The embryos were subjected to Immunocytochemistry using anti-BrdU antibody (1:100 dilution) and Alexa flour 488-conjugated anti-mouse IgG secondary antibody (1:100 dilution; Sigma).

Immunocytochemistry

The embryos were fixed in PBS containing 2% paraformaldehyde for 15 min at room temperature. The fixed embryos were permeabilized in 0.5% Triton X-100 diluted in PBS for 15 min at room temperature. After the embryos were washed with PBS containing 1% BSA (Sigma) at three times, the permeablized embryos were incubated with anti-phosphorylated Ser2 of RNA polymerase II antibody (Abcam, Cambridge,

UK) in PBS containing 1% BSA (1:100 dilution) for 1 hour at room temperature. The embryos were washed with PBS containing 1% BSA and incubated with FITC (fluorescein)-conjugated anti-mouse IgM secondary antibody (1:100 dilution; Abcam) for 1 hour at room temperature. The embryos were mounted on slide glass using VectaShield (Vector Laboratories, Burlingame, CA, USA) supplemented with 3 mg/ml 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Kumamoto, Japan). Confocal digital images were collected using a confocal laser-scanning microscope (LSM 5 EXCITER: Carl Zeiss MicroImaging GmbH, Oberkochen).

RNA extraction and preparation of the RNA-seq library

C57BL/6J mice of oocytes and embryos were subjected to RNA extraction for RNA sequencing. Total RNA was extracted from 3,000 of oocytes, 3,000 of 1-cell stage embryos, 4,500 of 2-cell stage embryos, 2,800 of 4-cell stage embryos, 1,400 of morulas and 700 of blastocysts, using Isogen (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. The quality of RNA was verified on the Bioanalyzer RNA Pico Chip (Agilent Technologies, Santa Clara, CA). RNA-Seq libraries were constructed using the mRNA-Seq Sample Preparation Kit (Illumina, San Diego, CA) without selection of polyadenylated RNA.

Sequencing and mapping of RNA-seq reads on the mouse genome

RNA-seq libraries were subjected to sequencing using Genome Analyzer IIx (Illumina). The 35-nt single-end sequencing reads were mapped onto the mouse genome reference sequence (mm9, UCSC Genome Browser) allowing for no mismatch using the ELAND software (Table 1). The uniquely mapped reads on RefSeq genes were used to calculate

RPKM values of RefSeq genes in each sample. The results of RNA-seq data analysis were visualized on the genome browser using the Genome Studio software (Illumina).

To analyze intergenic regions, 76-nt paired-end-sequencing reads were mapped onto mouse genome reference sequences (mm9) allowing 1- or 2-nt mismatches using the GSNAP software (Wu and Nacu, 2010). These mapped 76-nt reads (excluding those on rRNA and tRNA genes) were used to construct sequence fragments for each pair of reads (Table 2). To adjust the number of fragments used for sample analysis, 18 million fragments were randomly selected from each sample. To collect the fragments that mapped to intergenic regions, we removed fragments that overlapped with gene regions annotated in RefSeq and Ensemble.

k-mean nonhierarchical clustering

Relative expression levels were calculated by RPKM values in oocytes, 1- and 2-cell stage embryos. The average RPKM values between oocytes, 1- and 2-cell stage embryos were set as 1. The relative expression values were converted into log10 values and subjected to k-mean nonhierarchical clustering using TIGR Multiple Experiment Viewer (MeV) (Saeed et al., 2003).

Reverse transcription and polymerase chain reaction

Total RNA was isolated from 100 oocytes and embryos using Isogen (Nippon Gene), and was prepared for reverse-transcription using a PrimeScript RT-PCR kit (Takara Bio Inc., Otsu), according to the manufacturer’s instructions. PCR was performed in a thermal cycler (iCycler; Bio-Rad, Berkeley, CA) using Ex Taq DNA polymerase (Takara) and following described primer pairs: intergenic-1:

5’-GCATTGTCCCTTGATTCAGC-3’ and 5’-CCAAGCAAGGCTTTGTGAA-3’, intergenic-2: 5’-ACCCTCAAGCACCTTTCCTT-3’ and 5’-AGCAGATGCCCAGCATAAAC-3’, linc-1: 5’-CAGGCATTGCACAACTCAAG-3’ and 5’-GAGCAACGAGAGCCAAGTTT-3’, linc-2: 5’-CCTGAACAACAAAAGCAGCA-3’ and 5’-GCTTCAGGCTGGTAGACTGG-3’, linc-3: 5’-AGTGCCTTGCTGCATTGTTCATCT-3’ and 5’-TGCCAAGAGGCTGGCTTCCTA-3’, linc-4: 5’-TAATGTCACATGCCCTTGGA-3’ and 5’-GGGCACTTAGCAAGAACCAC-3’, *GAPDH*: 5’-ACCACAGTCCATGCCATCAC-3’ and 5’-TCCACCACCCTGTTGCTGTA-3’, and Rabbit *α-globin*: 5’-GTGGGACAGGAGCTTGAAAT-3’ and 5’-GCAGCCACGGTGGCGAGTAT-3’. The PCR conditions were following programs: *GAPDH*: 32 cycles of 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 60 s. Rabbit *α-globin*: 26 cycles of 95ºC for 30 s, 58ºC for 30 s, and 72ºC for 60 s. Other primers: 38 cycles of 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 60 s.

Microinjection of LNA-DNA gapmers

The embryos were transfer to HEPES-buffered KSOM between 2 and 4 h after insemination and injected into the cytoplasm of 1-cell stage embryos with LNA-DNA gapmers which were diluted in nuclease-free water to 10 μM. LNA-DNA gapmers were purchased from Gene Design (Osaka, Japan): the sequences were as shown in Table 3.

Sequence information of core promoter elements and collection of G/C-rich regions in intergenic regions

Sequences of core promoter elements were referred to (Smale and Kadonaga, 2003).

G/C-rich regions in intergenic region were collected from the list of 50 bp sequences that were created by the 50 bp sliding window analysis with a 25 bp-shift. The regions of over 60% G/C contents in the list of 50 bp sequences were categorized as G/C-rich regions. Refseq and Ensemble annotated genes, CpG islands and these 2 kb up- and downstream regions were removed from the list of G/C-rich regions. Next to or overlapped G/C-rich regions with each others were regarded as one region and combined with each other. When G/C-rich regions were mapped within 1 kb of each other, these regions were removed from the list of G/C-rich regions.

Tables

Table 1. Number of uniquely mapped fragments in 36-nt single end sequences

	Raw read	Uniquely mapped	
		Mapped to whole genome	Mapped to Refseq
Oocyte	43,269,123	7,487,627	6,706,633
1-cell	48,763,125	6,758,142	5,871,456
2-cell	37,025,705	3,989,555	3,367,986
2-cell +DRB[a]	29,600,507	10,255,740	6,454,111

[a] Embryos were treated with 5, 6-dichlorobenzimidazole riboside (DRB) between 1-cell stage and G1 phase of 2-cell stage.

Table 2. Number of uniquely mapped fragments in 76-nt paired end sequences

Oocyte	25,293,937
1-cell	28,667,882
2-cell	24,992,645
4-cell	28,495,811
Morula	30,883,015
Blastocyst	31,979,743
1-cell +DRB[a]	18,179,217
2-cell +Aph.[b]	25,211,912

[a] Embryos were treated with 5, 6-dichlorobenzimidazole riboside (DRB) during one-cell-stage.

[b] Embryos were treated with aphidicolin during two-cell-stage.

Table 3. Sequence of LNA-DNA gapmers

Non-targeting	GCGCgctttgtaggaTTCG
Not expressed region	GATGgattttaaacCATC
linc1-A	AACTattcaagtttCCTG
linc1-B	ACCAactattcaagTTTC
linc2-A	GTAGactgggtttgAGTG
linc2-B	ACCCttccaagtgaTTTC
linc3-A	GAACactgagtacaTGAC
linc3-B	ATGActgagagtgaGCAC
linc4-A	TACTattctaaggtCTCA
linc4-B	ATTCtaaggtctcaCAAA

LNA nucleotides represent uppercase letters

References

ABE, K., INOUE, A., SUZUKI, M.G., and AOKI, F. (2010). Global gene silencing is caused by the dissociation of RNA polymerase II from DNA in mouse oocytes. J Reprod Dev *56*, 502-507.

Ahmed, K., Dehghani, H., Rugg-Gunn, P., Fussner, E., Rossant, J., and Bazett-Jones, D.P. (2010). Global chromatin architecture reflects pluripotency and lineage commitment in the early mouse embryo. PLoS One *5*, e10531.

Akiyama, T., Suzuki, O., Matsuda, J., and Aoki, F. (2011). Dynamic replacement of histone H3 variants reprograms epigenetic marks in early mouse embryos. PLoS Genet *7*, e1002279.

Aoki, F., Worrad, D.M., and Schultz, R.M. (1997). Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. Dev Biol *181*, 296-307.

Beaujean, N., Bouniol-Baly, C., Monod, C., Kissa, K., Jullien, D., Aulner, N., Amirand, C., Debey, P., and Käs, E. (2000). Induction of early transcription in one-cell mouse embryos by microinjection of the nonhistone chromosomal protein HMG-I. Dev Biol *221*, 337-354.

Bensaude, O., Babinet, C., Morange, M., and Jacob, F. (1983). Heat shock proteins, first major products of zygotic gene activity in mouse embryo. Nature *305*, 331-333.

Bergmann, J.H., and Spector, D.L. (2014). Long non-coding RNAs: modulators of nuclear structure and function. Curr Opin Cell Biol *26*, 10-18.

Bolton, V.N., Oades, P.J., and Johnson, M.H. (1984). The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo. J Embryol Exp Morphol *79*, 139-163.

Braasch, D.A., and Corey, D.R. (2001). Locked nucleic acid (LNA): fine-tuning the

recognition of DNA and RNA. Chem Biol *8*, 1-7.

Cabili, M.N., Trapnell, C., Goff, L., Koziol, M., Tazon-Vega, B., Regev, A., and Rinn, J.L. (2011). Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev *25*, 1915-1927.

Cao, M. et al. (2013). Department of Integrated Biosciences Graduate School of Frontier Science, The university of Tokyo. Master thesis.

Cho, T., Sakai, S., Nagata, M. and Aoki, F. (2002) Involvement of chromatin structure in the regulation of mouse zygotic gene activation. Anim. Sci. J., 73, 113-122.

Cesana, M., Cacchiarelli, D., Legnini, I., Santini, T., Sthandier, O., Chinappi, M., Tramontano, A., and Bozzoni, I. (2011). A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell *147*, 358-369.

Chodosh, L.A., Fire, A., Samuels, M., and Sharp, P.A. (1989). 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II in vitro. J Biol Chem *264*, 2250-2257.

Cieśla, M., Dulak, J., and Józkowicz, A. (2014). MicroRNAs and epigenetic mechanisms of rhabdomyosarcoma development. Int J Biochem Cell Biol.

Conover, J.C., Temeles, G.L., Zimmermann, J.W., Burke, B., and Schultz, R.M. (1991). Stage-specific expression of a family of proteins that are major products of zygotic gene activation in the mouse embryo. Dev Biol *144*, 392-404.

Davis, W., De Sousa, P.A., and Schultz, R.M. (1996). Transient expression of translation initiation factor eIF-4C during the 2-cell stage of the preimplantation mouse embryo: identification by mRNA differential display and the role of DNA replication in zygotic gene activation. Dev Biol *174*, 190-201.

Dogini, D.B., Pascoal, V.D., Avansini, S.H., Vieira, A.S., Pereira, T.C., and Lopes-Cendes, I. (2014). The new world of RNAs. Genet Mol Biol *37*, 285-293.

Dubois, M.F., Bellier, S., Seo, S.J., and Bensaude, O. (1994). Phosphorylation of the

RNA polymerase II largest subunit during heat shock and inhibition of transcription in HeLa cells. J Cell Physiol *158*, 417-426.

Fenouil, R., Cauchy, P., Koch, F., Descostes, N., Cabeza, J.Z., Innocenti, C., Ferrier, P., Spicuglia, S., Gut, M., Gut, I., *et al.* (2012). CpG islands and GC content dictate nucleosome depletion in a transcription-independent manner at mammalian promoters. Genome research *22*, 2399-2408.

Flach, G., Johnson, M.H., Braude, P.R., Taylor, R.A., and Bolton, V.N. (1982). The transition from maternal to embryonic control in the 2-cell mouse embryo. EMBO J *1*, 681-686.

Forlani, S., Bonnerot, C., Capgras, S., and Nicolas, J.F. (1998). Relief of a repressed gene expression state in the mouse 1-cell embryo requires DNA replication. Development *125*, 3153-3166.

Grote, P., Wittler, L., Hendrix, D., Koch, F., Währisch, S., Beisaw, A., Macura, K., Bläss, G., Kellis, M., Werber, M., *et al.* (2013). The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. Dev Cell *24*, 206-214.

Guttman, M., Amit, I., Garber, M., French, C., Lin, M.F., Feldser, D., Huarte, M., Zuk, O., Carey, B.W., Cassady, J.P., *et al.* (2009). Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature *458*, 223-227.

Hamatani, T., Carter, M.G., Sharov, A.A., and Ko, M.S. (2004). Dynamics of global gene expression changes during mouse preimplantation development. Dev Cell *6*, 117-131.

Henery, C.C., Miranda, M., Wiekowski, M., Wilmut, I., and DePamphilis, M.L. (1995). Repression of gene expression at the beginning of mouse development. Dev Biol *169*, 448-460.

Hutchins, J.R., and Clarke, P.R. (2004). Many fingers on the mitotic trigger: post-translational regulation of the Cdc25C phosphatase. Cell Cycle *3*, 41-45.

Hutchinson, J.N., Ensminger, A.W., Clemson, C.M., Lynch, C.R., Lawrence, J.B., and Chess, A. (2007). A screen for nuclear transcripts identifies two linked noncoding RNAs associated with SC35 splicing domains. BMC Genomics *8*, 39.

Kageyama, S., Gunji, W., Nakasato, M., Murakami, Y., Nagata, M., and Aoki, F. (2007). Analysis of transcription factor expression during oogenesis and preimplantation development in mice. Zygote *15*, 117-128.

Klattenhoff, C.A., Scheuermann, J.C., Surface, L.E., Bradley, R.K., Fields, P.A., Steinhauser, M.L., Ding, H., Butty, V.L., Torrey, L., Haas, S., *et al.* (2013). Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. Cell *152*, 570-583.

Kong, L., Zhang, Y., Ye, Z.Q., Liu, X.Q., Zhao, S.Q., Wei, L., and Gao, G. (2007). CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. Nucleic Acids Res *35*, W345-349.

Larochelle, S., Amat, R., Glover-Cutter, K., Sansó, M., Zhang, C., Allen, J.J., Shokat, K.M., Bentley, D.L., and Fisher, R.P. (2012). Cyclin-dependent kinase control of the initiation-to-elongation switch of RNA polymerase II. Nat Struct Mol Biol *19*, 1108-1115.

Lawinger, P., Rastelli, L., Zhao, Z., and Majumder, S. (1999). Lack of enhancer function in mammals is unique to oocytes and fertilized eggs. J Biol Chem *274*, 8002-8011.

Lawitts, J.A., and Biggers, J.D. (1993). Culture of preimplantation embryos. Methods in enzymology *225*, 153-164.

Levey, I. L. and Brinster, R. L. (1978). Effect of a-amanitin on RNA synthesis by mouse embryos in culture. J. Exp. Zool *203*, 351-360.

Loewer, S., Cabili, M.N., Guttman, M., Loh, Y.H., Thomas, K., Park, I.H., Garber, M., Curran, M., Onder, T., Agarwal, S., *et al.* (2010). Large intergenic non-coding RNA-RoR modulates reprogramming of human induced pluripotent stem cells. Nat Genet *42*, 1113-1117.

Majumder, S., and DePamphilis, M.L. (1995). A unique role for enhancers is revealed during early mouse development. Bioessays *17*, 879-889.

Majumder, S., Miranda, M., and DePamphilis, M.L. (1993). Analysis of gene expression in mouse preimplantation embryos demonstrates that the primary role of enhancers is to relieve repression of promoters. EMBO J *12*, 1131-1140.

Majumder, S., Zhao, Z., Kaneko, K., and DePamphilis, M.L. (1997). Developmental acquisition of enhancer function requires a unique coactivator activity. EMBO J *16*, 1721-1731.

Martin, C., Brochard, V., Migné, C., Zink, D., Debey, P., and Beaujean, N. (2006). Architectural reorganization of the nuclei upon transfer into oocytes accompanies genome reprogramming. Mol Reprod Dev *73*, 1102-1111.

Matsumoto, K., Anzai, M., Nakagata, N., Takahashi, A., Takahashi, Y., and Miyata, K. (1994). Onset of paternal gene activation in early mouse embryos fertilized with transgenic mouse sperm. Mol Reprod Dev *39*, 136-140.

Moore, G.P., Lintern-Moore, S., Peters, H., and Faber, M. (1974). RNA synthesis in the mouse oocyte. J Cell Biol *60*, 416-422.

Nashun, B., Yukawa, M., Liu, H., Akiyama, T., and Aoki, F. (2010). Changes in the nuclear deposition of histone H2A variants during pre-implantation development in mice. Development *137*, 3785-3794.

Nothias, J.Y., Majumder, S., Kaneko, K.J., and DePamphilis, M.L. (1995). Regulation of Gene Expression at the Beginning of Mammalian Development. J Biol Chem *270*, 22077-22080.

Ohashi, M., Taguchi, T., and Ikegami, S. (1978). Aphidicolin: a specific inhibitor of DNA polymerases in the cytosol of rat liver. Biochem Biophys Res Commun *82*, 1084-1090.

Park, S.J., Komata, M., Inoue, F., Yamada, K., Nakai, K., Ohsugi, M., and Shirahige, K. (2013). Inferring the choreography of parental genomes during fertilization from

ultralarge-scale whole-transcriptome analysis. Genes Dev *27*, 2736-2748.

Probst, A.V., Okamoto, I., Casanova, M., El Marjou, F., Le Baccon, P., and Almouzni, G. (2010). A strand-specific burst in transcription of pericentric satellites is required for chromocenter formation and early mouse development. Dev Cell *19*, 625-638.

Quinn, P., and Begley, A.J. (1984). Effect of human seminal plasma and mouse accessory gland extracts on mouse fertilization in vitro. Australian journal of biological sciences *37*, 147-152.

Raju, U., Koumenis, C., Nunez-Regueiro, M., and Eskin, A. (1991). Alteration of the phase and period of a circadian oscillator by a reversible transcription inhibitor. Science *253*, 673-675.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., *et al.* (2003). TM4: a free, open-source system for microarray data management and analysis. Biotechniques *34*, 374-378.

Sandelin, A., Carninci, P., Lenhard, B., Ponjavic, J., Hayashizaki, Y., and Hume, D.A. (2007). Mammalian RNA polymerase II core promoters: insights from genome-wide studies. Nature reviews Genetics *8*, 424-436.

Schultz, R.M. (1993). Regulation of zygotic gene activation in the mouse. Bioessays *15*, 531-538.

Smale, S.T., and Kadonaga, J.T. (2003). The RNA polymerase II core promoter. Annu Rev Biochem *72*, 449-479.

Tam, O.H., Aravin, A.A., Stein, P., Girard, A., Murchison, E.P., Cheloufi, S., Hodges, E., Anger, M., Sachidanandam, R., Schultz, R.M., *et al.* (2008). Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. Nature *453*, 534-538.

Toyoda Y, Yokoyama M, Hosi T. (1971). Studies on the fertilization of mouse eggs in vitro: I. in vitro fertilization of eggs by fresh epididymal sperm. The Japanese journal of animal reproduction **16:** 147-151.

van Blerkom, J., and Brockway, G.O. (1975). Qualitative patterns of protein synthesis in the preimplantation mouse embryo. I. Normal pregnancy. Dev Biol *44*, 148-157.

Wang, P., Xue, Y., Han, Y., Lin, L., Wu, C., Xu, S., Jiang, Z., Xu, J., Liu, Q., and Cao, X. (2014). The STAT3-binding long noncoding RNA lnc-DC controls human dendritic cell differentiation. Science *344*, 310-313.

Wang, Q., Su, L., Liu, N., Zhang, L., Xu, W., and Fang, H. (2011). Cyclin dependent kinase 1 inhibitors: a review of recent progress. Curr Med Chem *18*, 2025-2043.

Wang, Q.T., Piotrowska, K., Ciemerych, M.A., Milenkovic, L., Scott, M.P., Davis, R.W., and Zernicka-Goetz, M. (2004). A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. Dev Cell *6*, 133-144.

Warner, C.M., and Versteegh, L.R. (1974). In vivo and in vitro effect of alpha-amanitin on preimplantation mouse embryo RNA polymerase. Nature *248*, 678-680.

Wiekowski, M., Miranda, M., and DePamphilis, M.L. (1991). Regulation of gene expression in preimplantation mouse embryos: effects of the zygotic clock and the first mitosis on promoter and enhancer activities. Dev Biol *147*, 403-414.

Wiekowski, M., Miranda, M., and DePamphilis, M.L. (1993). Requirements for promoter activity in mouse oocytes and embryos distinguish paternal pronuclei from maternal and zygotic nuclei. Dev Biol *159*, 366-378.

Wolgemuth, D.J. (2011). Function of the A-type cyclins during gametogenesis and early embryogenesis. Results Probl Cell Differ *53*, 391-413.

Wu, T.D., and Nacu, S. (2010). Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics *26*, 873-881.

Xiong, Y., Chen, X., Chen, Z., Wang, X., Shi, S., Zhang, J., and He, X. (2010). RNA sequencing shows no dosage compensation of the active X-chromosome. Nat Genet *42*, 1043-1047.

Xue, Z., Huang, K., Cai, C., Cai, L., Jiang, C.Y., Feng, Y., Liu, Z., Zeng, Q., Cheng, L., Sun, Y.E., *et al.* (2013). Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature *500*, 593-597.

Yamamoto, R. et al. (2012). Department of Integrated Biosciences Graduate School of Frontier Science, The university of Tokyo. Master thesis.

Yoon, J.H., Abdelmohsen, K., Kim, J., Yang, X., Martindale, J.L., Tominaga-Yamanaka, K., White, E.J., Orjalo, A.V., Rinn, J.L., Kreft, S.G., *et al.* (2013). Scaffold function of long non-coding RNA HOTAIR in protein ubiquitination. Nat Commun *4*, 2939.

Yu, J., Hecht, N.B., and Schultz, R.M. (2003). Requirement for RNA-binding activity of MSY2 for cytoplasmic localization and retention in mouse oocytes. Dev Biol *255*, 249-262.

Zandomeni, R., Zandomeni, M.C., Shugar, D., and Weinmann, R. (1986). Casein kinase type II is involved in the inhibition by 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole of specific RNA polymerase II transcription. J Biol Chem *261*, 3414-3419.

Zhao, J., Ohsumi, T.K., Kung, J.T., Ogawa, Y., Grau, D.J., Sarma, K., Song, J.J., Kingston, R.E., Borowsky, M., and Lee, J.T. (2010). Genome-wide identification of polycomb-associated RNAs by RIP-seq. Mol Cell *40*, 939-953.