平成 21 年度　博士論文

Doctoral Dissertation

PGC7/Stella is required for the condensation of chromatin in full grown oocyte

（PGC7/Stella が成長卵におけるクロマチンの凝縮及び発生能に及ぼす影響）

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Abstract

Mammalian oocytes have two types of full-grown oocytes (FGOs) based on their chromatin organization. One is surrounded nucleolus (SN)-type oocytes in which the chromatin is condensed and surrounds the nucleolus, and the other is non-surrounded nucleolus (NSN)-type oocytes in which the chromatin is less condensed and does not surround the nucleolus. In addition, SN-type oocytes can develop to the blastocyst stage after fertilization, whereas NSN-type oocytes can not. We have been analyzing the function of PGC7 (also known as Stella, Dppa3) and revealed that PGC7/stella is required for protection of maternal genome from active DNA demethylation in fertilized egg. However, the functions of PGC7/stella before fertilization remain unknown.

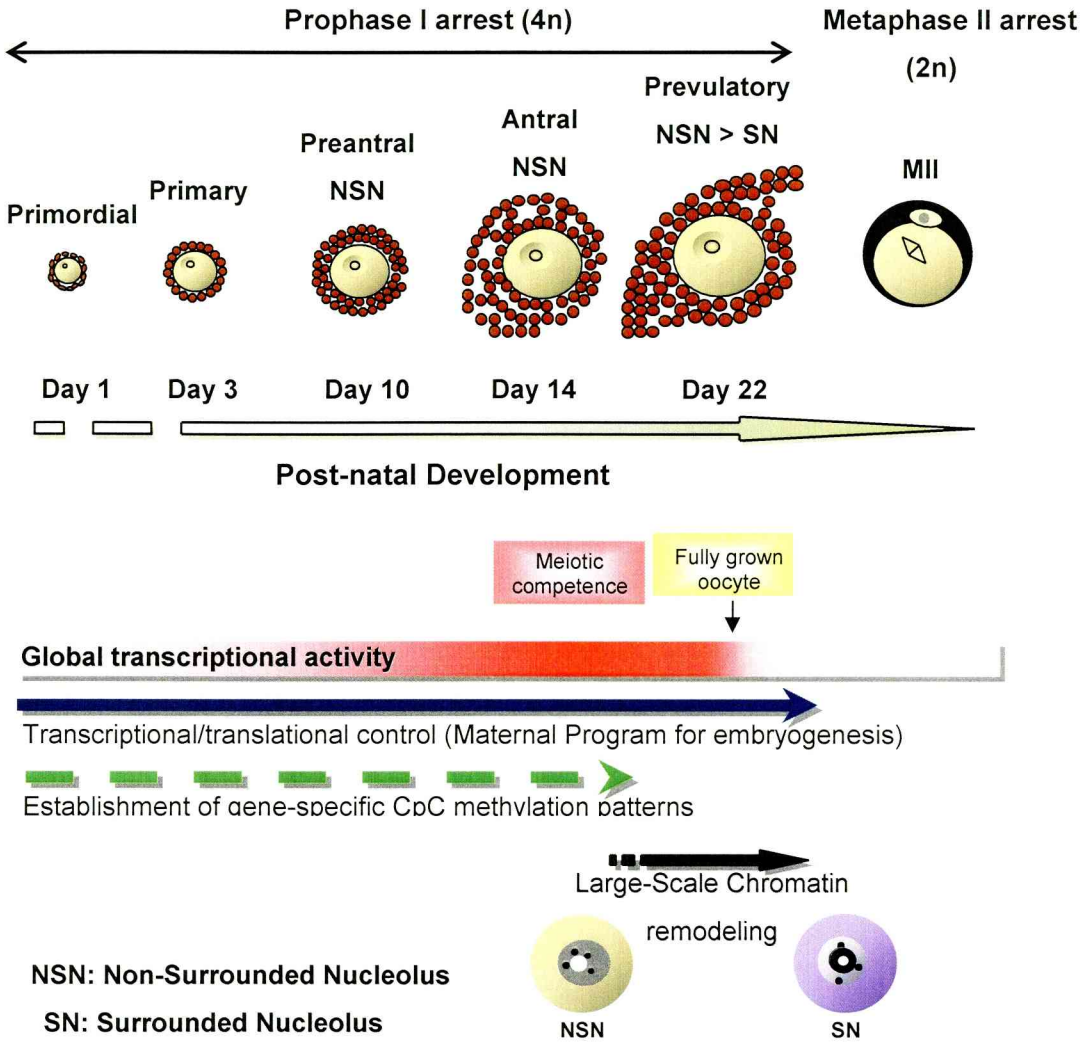
In this study, I analyzed the function of PGC7/stella during oocyte growth. First, I analyzed the localization of PGC7/stella in FGOs and found that PGC7/stella was localized in the nucleus around the nucleolus in only SN-type oocytes. Next, I examined the chromatin configuration of FGOs in PGC7/stella null ovary. Although 63% of wild-type FGOs were SN-type oocytes, less than 20% of PGC7/stella null FGOs were SN-type oocytes. PGC7/stella null FGOs had less condensed chromatin and did not surround the nucleolus similar to that of NSN-type oocytes. Since the global epigenetic modification of NSN-type oocytes was different from that in SN-type oocytes, I analyzed the epigenetic status of PGC7/stella null FGOs. The results revealed that the extent of epigenetic modification such as histone methylation was slightly decreased in PGC7/stella -null FGOs. My results suggested a novel role of maternal PGC7/stella which is involved in the chromatin organization with the onset of large-scale chromatin remodeling, epigenetic modification, leading to transition into the SN configuration and the global transcriptional repression during oocyte growth to acquire of developmental

competence.

Introduction

Functional differentiation of chromatin structure during mouse oocyte growth

In the mammalian neonatal ovary, oocytes are naturally arrest at prophase I of meiosis. From the onset of ovarian follicle activation, oocytes are maintained in a prolonged meiotic arrest at the dipotene or dictyate stage during postnatal development. Coordinated transcription and translational control mechanisms regulate single copy gene expression in the oocyte genome. Synthesis and storage of dormant maternal mRNAs during oogenesis are essential for the completion of meiosis and preimplantation development. During oocyte growth, maternal-specific imprints are established on a locus by locus basis (Bourc'his *et al.*, 2001; Lucifero *et al.*, 2004; Obata *et al.*, 2002). Coincident with the formation of antral follicles on day 14 of post-natal development, oocytes acquire meiotic competence. At this stage, the oocyte genome is at the peak of its global transcriptional activity. Initially, all oocytes are in the non-surrounded nucleolus (NSN) configuration, which the chromatin is less condensed, and not surrounded the nucleolus but from day 17 of post-natal development onwards, subsequent oocyte growth and differentiation some oocyte continue their development in the NSN configuration and some are shifted into the surrounded nucleolus (SN) configuration in which the chromatin is highly condensed and is concentrated around the nucleolus. The change of the chromatin configuration is associated with the onset of large-scale chromatin remodeling and global transcriptional quiescence in a cohort of pre-ovulatory oocytes (Fig.1). However the mechanisms regulating large-scale chromatin remodeling in the germinal vesicle (GV) are not known.



Modified from R. De La Fuente /Developmental biology 292 1–12 (2006)

Figure 1. Functional differentiation of chromatin structure during mouse oocyte growth

Coordinated transcription and translational control mechanisms regulate gene expression in the oocyte genome. During oocyte growth, maternal-specific imprints are established. Coincident with the formation of antral follicles on day 14 after birth, oocytes acquire meiotic competence. At this stage, the oocyte genome is at the peak of its global transcriptional activity. From day 17 after birth, oocyte growth and differentiation are associated with the onset of large-scale chromatin remodeling, leading to the transition into the SN configuration and global transcriptional repression in pre-ovulatory oocytes

The types of fully grown oocyte

In the mammalian ovary, oocytes during growth and differentiation, most of fully grown oocytes (FGOs) undergo a dramatic change in nuclear organization in which chromatin become progressively condensed, however some FGOs do not. Thus, mammalian oocytes have two types of FGOs based on their chromatin organization. One is surrounded nucleolus (SN)-type oocytes in which the chromatin is condensed and surrounds the nucleolus, and the other is non-surrounded nucleolus (NSN)-type oocytes in which the chromatin is less condensed and does not surround the nucleolus (Mattson and Albertini 1990; Debey *et al.*, 1993) (Fig. 2). In addition to a differential chromatin configuration, other differences have been reported between SN- and NSN-type oocytes in mice. For instance, the microtubule organizing centers (MTOCs) form around the germinal vesicles (GV) of SN-type oocytes, whereas no MTOCs form in NSN-type oocytes (Wickramasinghe and Albertini 1992; Can *et al.*, 2003). The structure of the nucleolus is vacuolated in NSN-type and compact in SN-type oocytes (Debey *et al.*, 1993). Furthermore, SN- and partial SN-type oocytes were silent in relation to polI- and pol II-dependent transcription, while NSN-type oocytes were actively transcribed. (BouniolBaly *et al.*, 1999; Miyara *et al.*, 2003). The extent of epigenetic modification, such as DNA methylation, histone methylation and acetylation is higher in SN oocytes (Kageyama *et al.*, 2007). Moreover, when the two types of oocyte are matured to MII oocyte, only SN-type oocytes can develop to the blastocyst stage after fertilization, whereas NSN-type oocytes can not (Zuccotti *et al.*, 2002; Inoue *et al.*, 2008). Thus, several cytoplasmic, nuclear and epigenetic properties differ between SN and NSN oocytes, however the factors involved in coordinating dynamic changes in large chromatin with the onset of transcriptional repression are poorly understood.

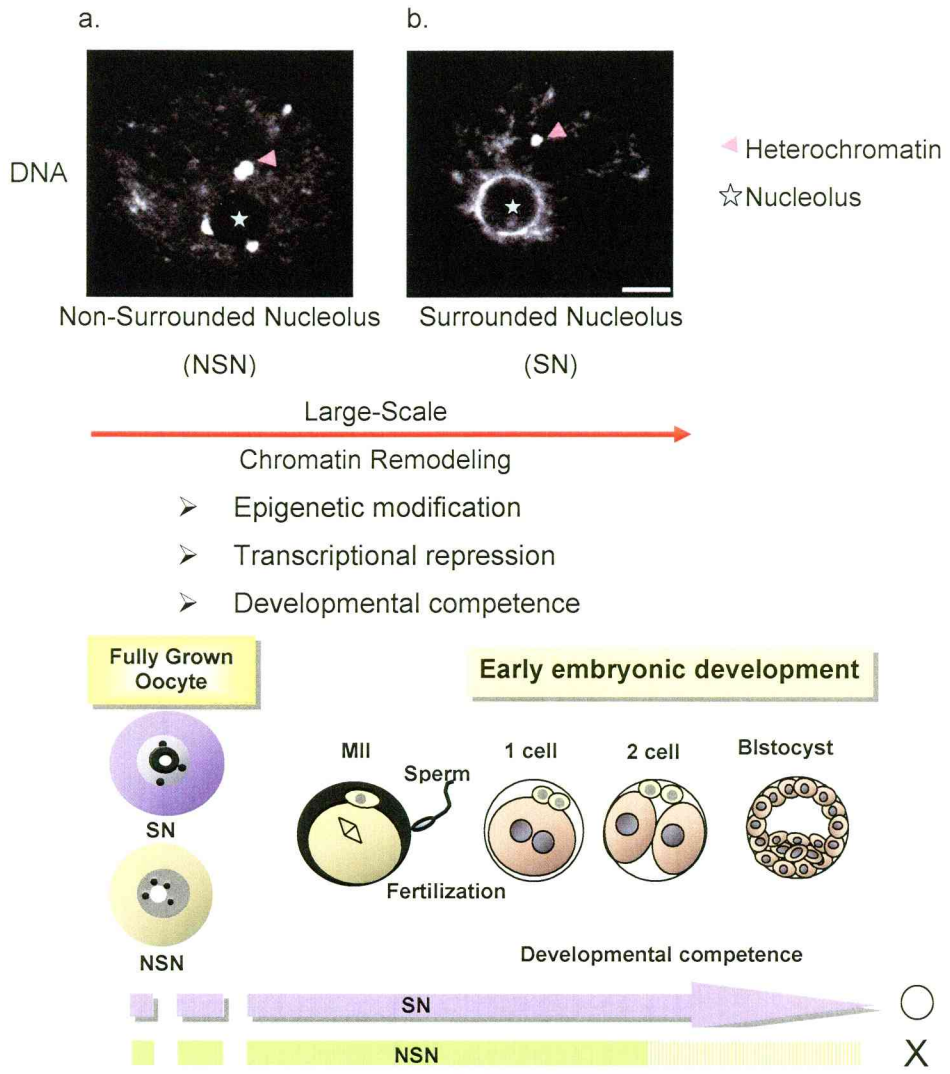


Figure 2 Chromatin configuration in the germal vescle(GV) of fully grown mouse oocyte

a. A decondensed chromatin configuration (Non-surrounded nucleolus; NSN) with prominent heterochromatin regions (arrowhead). Nucleolus is indicated by (*).DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and shown in white.

b. Chromatin condensation around the nucleolus (Surrounded nucleolus; SN) is associated with formation of a heterochromatin rim.

Large-scale chromatin remodeling events associated with global epigenetic modification, transcription repression and developmental competence.

Scale bar=10 μm

The function and expression pattern of PGC7/stella

PGC7/stella was initially cloned as a gene preferentially expressed in primordial germ cells (PGCs), after comparing gene expression in PGCs and embryonic stem (ES) cells, and single nascent germ cells and their somatic neighbors. Expression of PGC7/stella starts during the process of germ cell specification at embryonic day 7.25 (E7.25) specifically in the founder population of PGCs, and continues until E13.5 in female gonads and E15.5 in male gonads. Subsequently, no expression is detected in male mice. In contrast, PGC7/stella expression resumes in the immature oocytes and is maintained continuously until the oocytes mature (Fig. 3). Motif analysis of PGC7/stella suggested that it was localized in both the nucleus and cytoplasm and functions in DNA binding and RNA splicing (Fig. 4). The physiological function of PGC7/stella was analyzed in a gene disruption study which found that the gene was a maternal effect gene involved in early development, but not in germ cell development. We have been analyzing the function of PGC7/stella in detail and revealed that PGC7/stella is required for protection of maternal genome from active DNA demethylation in fertilized egg (Nakamura *et al.*, 2007) (Fig. 5). However, the functions of PGC7/stella before fertilization remain unclear. In this study, I analyzed the function of PGC7 in FGOs.

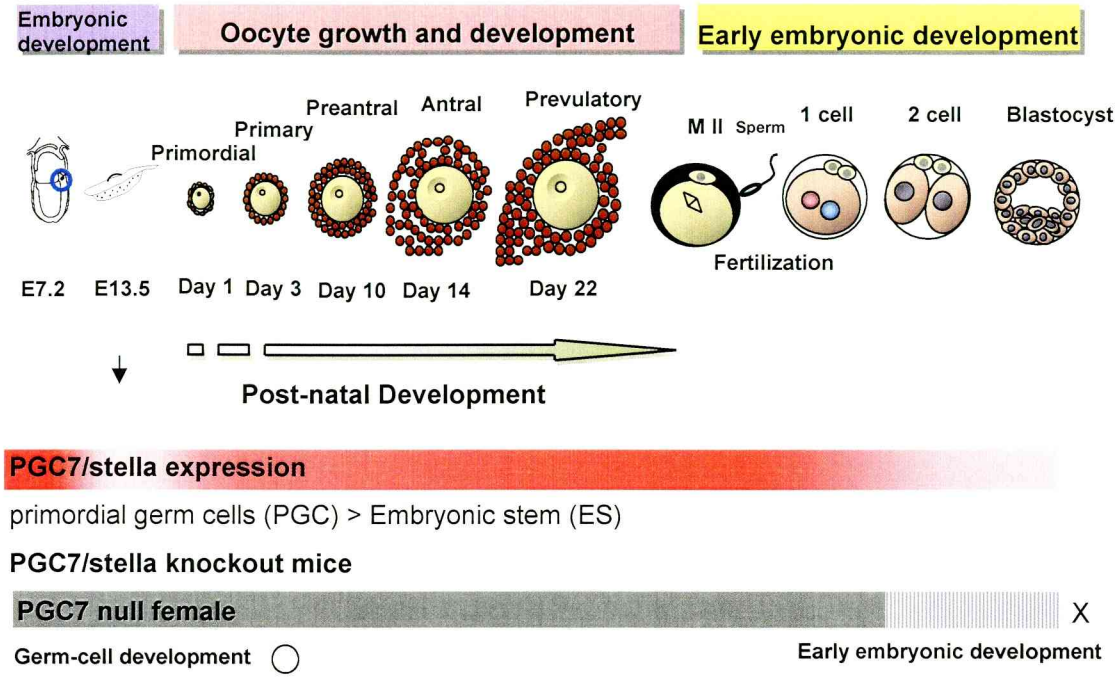
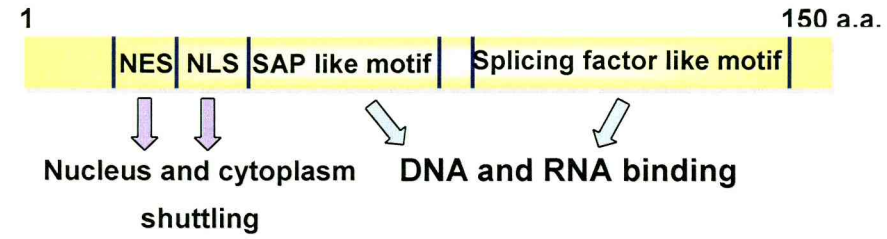


Figure 3 The expression pattern of PGC7/stella

Expression of PGC7 starts during the process of germ cell specification at embryonic day 7.25 (E7.25) specifically in the founder population of PGCs, and continues until E13.5 in female gonads. PGC7/stella expression resumes in the immature oocytes in the ovaries of newborn female mice and is maintained continuously until the oocytes mature. After fertilization, PGC7 expression persists in preimplantation embryos. The physiological function of PGC7 was analyzed in a gene disruption study which found that the gene was a maternal effect gene involved in early development, coursed female infertility, but not affect germ cell development.



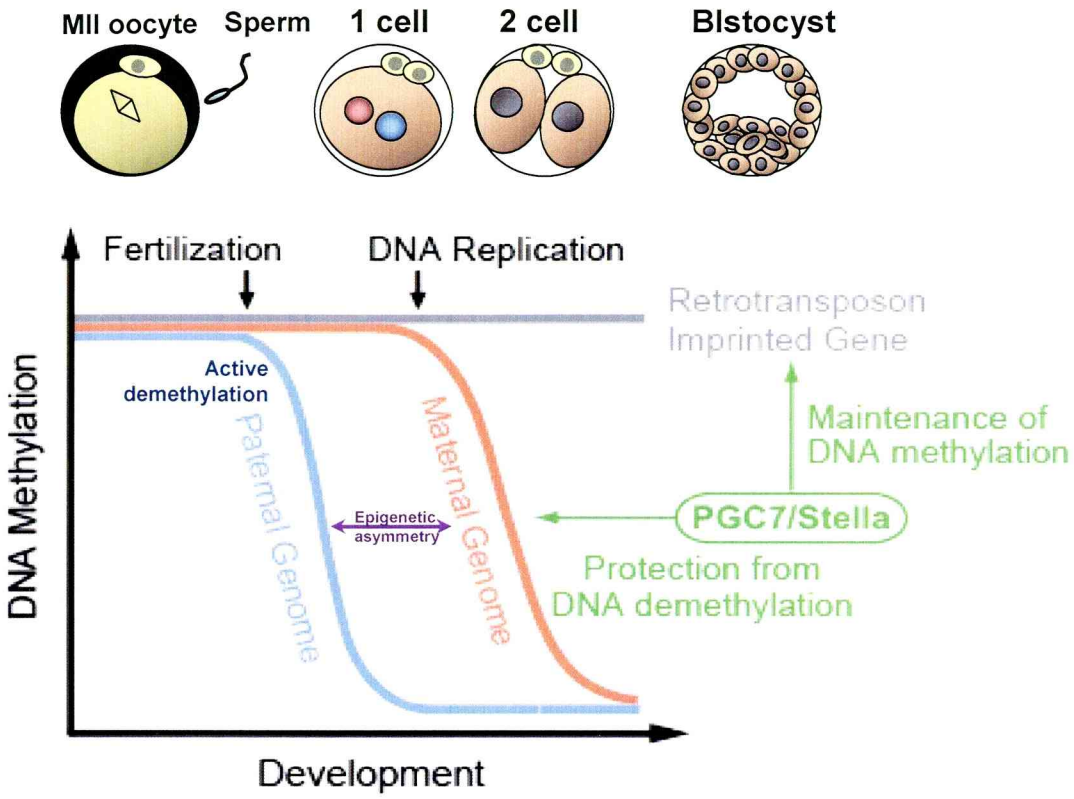
NLS: nuclear localization signal

NES: nuclear export signal

SAP: SAP-A/B, Acinus and PIAS

Figure 4 The motif analysis of PGC7/stella

PGC7/Stella contains a putative nuclear localization signal (NLS) and a nuclear export signal (NES), a SAP-like motif in N-terminal and a splicing factor-like motif in C-terminal suggested that it was localized in both the nucleus and cytoplasm and functions in DNA binding and RNA splicing, involved in DNA organization and RNA processing.



(Nakamura 2007)

Figure5. Functions of PGC7 in zygote

DNA methylation is an important means of epigenetic gene regulation and must be carefully controlled as a prerequisite for normal early embryogenesis. Although global demethylation occurs soon after fertilization, it is not evenly distributed throughout the genome. Genomic imprinting and epigenetic asymmetry between parental genomes, i.e., delayed demethylation of the maternal genome after fertilization, are clear examples of the functional importance of DNA methylation. Previous study showed that PGC7/Stella, a maternal factor essential for early development, serves to protect the DNA methylation state of several imprinted loci and epigenetic asymmetry.

Materials and Methods

Collection of Fully Grown Ovarian Oocytes

Fully grown ovarian oocytes (GV oocytes) were obtained from the ovaries of 7–10weeks old PGC7 $^{-/-}$, PGC7 $^{+/-}$ and BDF1, as wild type female mice. The females were injected intraperitoneally with 7.5-IU of pregnant mares serogonadotrophin (PMSG, Folligon, Intervet, Netherlands). Forty eight hours later, the ovaries were removed from mice and placed in FHM HEPES buffered medium (Millipore, Billerica, MA, USA) supplemented with 100 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, St Louis, MO, USA) to prevent spontaneous maturation of isolated oocytes. GV oocytes were collected by puncturing the largest preovulatory follicles with a fine needle, and the cumulus cells were gently removed from the cumulus–oocyte complexes using a narrow-bore glass pipette. Only those oocytes with a diameter greater than 70 mm were used in the following experiments.

Classification of oocytes

The oocytes were classified as SN- or NSN-type as described previously (Inoue *et al.*, 2007). Briefly, GV-stage oocytes were incubated in α-minimum essential medium (α -MEM) (Gibco-BRL, Grand Island, NY, USA) containing IBMX, 5% fetal bovine serum (FBS; Sigma-Aldrich). After 1 hour of incubation, some of the cells exhibited a perivitelline space (PVS) Fig.6. A very high correlation has been shown to exist between PVS formation and chromatin configuration. More than 90% of oocytes that form a PVS have a GV with the SN-type configuration, while all of the oocytes that lack a PVS have a GV with the NSN-type configuration (Inoue *et al.*, 2007). NSN-type FGOs were used for transcriptional inhibition and PGC7 mRNA injection experiments.

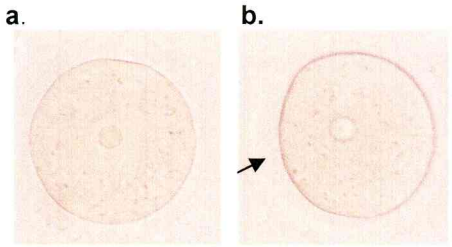


Figure 6 Formation of the pervitelline space (PVS) in mouse oocyte during *in vitro* culture with IBMX.

Light micrographs of fully grown oocytes with a PVS. The oocytes were observed after culture with IBMX for 1 hour. A PVS (arrow) was formed in the oocyte shown in panel b but not the in the one in panel a

Immunofluorescence confocal microscopy

Oocytes were fixed with 4% paraformaldehyde in PBS for 20 min. After washing with PBS/0.1% BSA, the oocytes were permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature and washed several times with PBS. The oocytes were blocking in 5% normal goat serum (Sigma-Aldrich) for 1 hour and incubated overnight at 4 ℃ with antibodies against PGC7 (1:10000; Sato *et al.*, 2002), histone H4 acetylated on lysines 5 and 12 (1:200; Upstate Biotechnology, Charlottesville, VA,USA), histone H3 di-methylated on lysine 4 (1:200; Upstate Biotechnology), histone H3 tri-methylated on lysine 4 (1:200; Abcam, Cambridge, MA, USA), histone H3 di-methylated or tri-methylated on lysine 9 (Abcam), and anti-RNA polymerase II phosporylated on serine 5 (1:200; H14:MMS-134R,Covance, Princeton NJ,USA). The oocytes were incubated with Alexa568-conjugated anti-rabbit-Ig (H+L) antibody or Alex488 anti-mouse Ig(H+L) antibody (1:500; Molecular Probes, Eugene, OR), at room temperature for 60 min. To visualize the DNA, the cells were counterstained with 1

μg/ml 4',6-diamidino-2-phenylindole (DAPI). The oocytes were mounted on glass slides and observed under the Carl Zeiss 510 laser-scanning confocal microscope (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany).

Detection of Transcription

Transcriptional activity was determined after 5-bromouridine 5'-triphosphate (BrUTP) incorporation as previously described (Bouniol *et al.* 1995). BrUTP (100mM solution in 2 mM Pipes buffered with 140 mM KCl, pH 7.4; Sigma-Aldrich) were microinjected into cytoplasm of GV oocytes, using an Eppendorf microinjector 5242 (Germany). After the injection, FGOs were cultured for 20 min in HEPES-buffered potassium simplex optimized medium (KSOM; Specialty Media, Phillipsburg, NJ, USA) with IBMX at 37 °C and then fixed for immunofluorescence. Incubation with the primary antibody (a mouse monoclonal antibody [IgG] raised against 5-bromo-2'-desoxyuridine (BrdU; Sigma-Aldrich) and recognizing BrU as well, diluted 1:100 in PBS/5% normal goat serum (Sigma-Aldrich) was performed overnight at 4 °C. After several times of wash with PBS/0.1% BSA, oocytes were treated with secondary antibody, Alexa568-conjugated anti-mouse-Ig(H+L) antibody (1:500) for 60 min. Oocytes were deposited on a slide, mounted, covered with coverslips, and examined with a Zeiss 510 laser-scanning confocal microscope.

Treatment with transcriptional inhibitor

FGOs were incubated in α-minimum essential medium (α-MEM) in the presence of 100 μM IBMX and 120 mM 5,6-dichlororibofuransyl-benzimidazole (DRB; Sigma-Aldrich) for 24 hours at 37 °C under 5% CO_2 and 95% air. After incubation, the oocytes were

stained with anti-PGC7 antibodies.

In vitro-transcribed PGC7 mRNA microinjection

Capped mRNA was made from NotI- or BamHI-linearized DNA constructs by in vitro transcription using a T7 mMessage mMachine kit (Ambion, Austin, TX, USA). Poly(A) tails were added to the capped mRNA using a Poly(A) Tailing Kit (Ambion) according to the manufacturer's instructions. To remove the template DNA, the reaction mixture was treated with Turbo DNase (provided with the in vitro transcription kit). The synthesized mRNA was purified by phenol/chloroform extraction and precipitate with ethanol. The mRNA samples were dissolved with nuclease free water to a final concentration of 100 ng/μl and stored at -80 °C until used. Microinjection was performed under an inverted microscope (Olympus IX-71, Olimpus Optical Co., Hamburg, Germany) using a micromanipulator and microinjector (both from Narishige Co., Tokyo, Japan). GV-stage oocytes were transferred to HEPES-buffered KSOM containing 100 μM IBMX and injected with ~10 pl of mRNA using narrow glass capillaries (Eppendorf microinjector 5242 Germany). Following microinjection, the oocytes were transferred into α-MEM containing 0.2 mM IBMX for 24 hours and then used for immunocytochemistry or immunoblotting.

In vitro transcribed PGC7 mRNA was microinjected into cytoplasm of PGC7/stella null FGOs, and then were incubated in α-MEM with 100 μM IBMX for 24 hours at 37 °C in 5% CO_2. After incubation, the oocytes were stained with anti-PGC7 antibody and DAPI.

Statistical analysis

Significance of the differences in percentage of NSN-, M-, and SN-type oocytes was assessed by chi-square analysis.

Results

Chromatin configuration of PGC7/stella null FGOs

The localization of PGC7/stella in FGOs was examined by immunocytochemistry. As shown in Fig. 7, PGC7/stella was diffusely localized in NSN-type oocytes. In contrast, its localization was concentrated around the nucleolus in SN-type oocytes. This result is consistent with previous reports showing the expression of PGC7/stella became progressively concentrated around the nucleolus (Zuccotti *et al.*, 2009). To directly assess whether PGC7/stella is required for the transition of NSN- to SN-type oocytes, chromatin configuration of PGC7/stella null FGOs were analyzed. Although 63% of wild-type FGOs were SN-type oocytes, less than 20% of PGC7/stella -null FGOs were SN-type oocytes. Furthermore, less than 46% of PGC7/stella heterozygous FGOs were SN-type oocytes. These data suggested that PGC7/stella is associated with the transition of NSN- to SN-type oocytes.

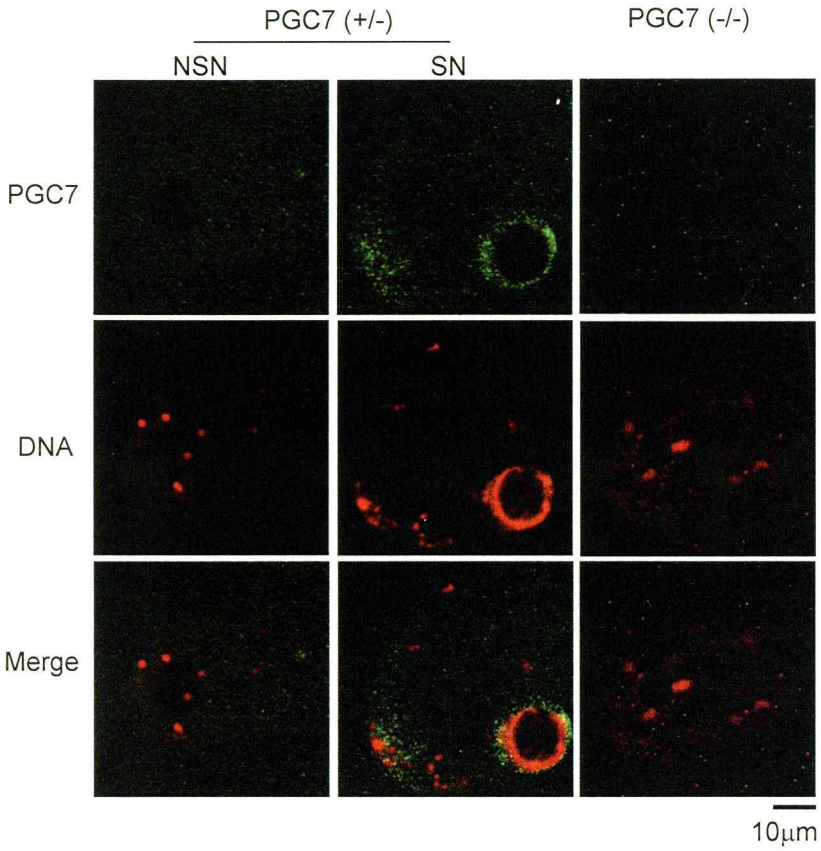


Figure 7 The localization of PGC7 in fully grown oocyte

$PGC7^{+/-}$ FGOs and $PGC7^{-/-}$ FGOs were immunostained with antibody against PGC7, which were showed in green. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and shown in red. SN-type oocytes (in which the condense heterochromatin is located around the nucleolus) and NSN-type oocytes (in which the heterochromatin does not surround the nucleolus) were distinguished according to chromatin configuration, as observed in the images of the DNA stained, FGOs. Confocal microscopy images of FGOs showed that compared with negative control, PGC7, shown in green was existed both cytoplasm and nucleus and was localized in the nucleus around the nucleolus in only SN-type oocytes. Scale bars=10 μm

Table 1

The proportion of chromatin configuration in fully grown oocytes derived from PGC7 $^{-/-}$, PGC7 $^{+/-}$ and WT mice ovary

Genotype of mice	Total no. oocytes	NSN	M	SN
WT	118	40 (33.9)	4 (3.3)	74(62.7)
PGC7 $^{+/-}$ [a]	186	71 (37.6)	29(15.4)	86 (45.6)
PGC7 $^{-/-}$ [a]	279	182(65.2)	42(15.1)	55(19.7)

(%) [a] The proportion of each type is significant different from wild type.

(Chi-square test<0.00001)

Figure 8

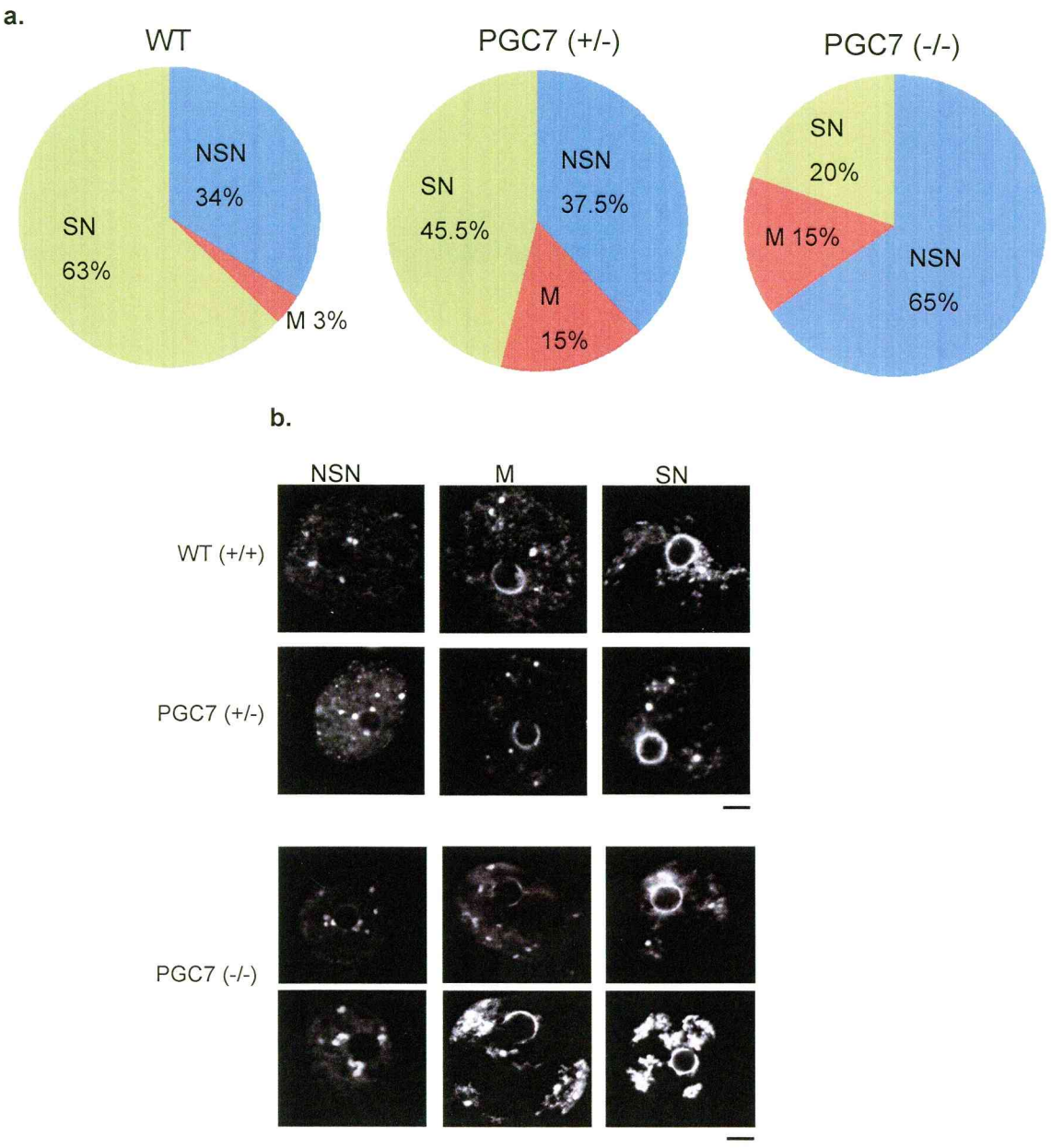


Figure 8 The chromatin configuration of PGC7 null fully grown oocytes

The FGOs were collected from 7-10 week old WT, $PGC7^{+/-}$ and $PGC7^{-/-}$ female mice and DNA were stained with 4',6-diamidino-2-phenylindole (DAPI) and shown in white. SN-type oocytes (in which the condense heterochromatin is located around the nucleolus) NSN-type oocytes (in which the heterochromatin does not surround the nucleolus), and intermediate type (M) which is with part of the chromatin beginning to make a ring around the nucleolus and part still dispersed were distinguished according to chromatin configuration, as observed in the images of the DNA stained

a. The percentages of SN- , NSN- and M- type chromatin configuration of WT, $PGC7^{+/-}$ and $PGC7^{-/-}$ were examined and shown in pie charts. The the chromatin configurations were significantly different between WT, $PGC7^{+/-}$and $PGC7^{-/-}$.

b. DNA was stained with DAPI and shown in white. The SN-, NSN-, and M- type chromatin configurations of WT, $PGC7^{+/-}$ and $PGC7^{-/-}$ FGOs was showed that the heterochromatin regions of $PGC7^{-/-}$ FGOs was some different from WT and $PGC7^{+/-}$ FGOs. Scale bars=10μm

The heterochromatin morphology of PGC7/stella null FGOs

The feature of NSN-type oocytes is the decondensed chromatin dispersed in nucleoplasm with several round prominent heterochromatin regions. Although all of PGC7/stella heterozygous NSN-type oocytes showed normal heterochromatin regions similar to that in wild-type NSN-type oocytes, about half of PGC7/stella null NSN-type oocytes showed abnormal heterochromatin regions (Fig. 9). Wild-type and PGC7/stella heterozygous NSN-type oocytes had condensed and round shape heterochromatin regions. However, irregular shape and less condensed heterochromatin regions were observed in PGC7/stella null NSN-type oocytes. In addition to NSN-type oocyte, abnormal chromatin configurations were observed in PGC7/stella null SN-type oocyte (Fig. 8b). Unlike NSN-type oocytes, few heterochromatin chromatin regions were observed in SN-type oocytes, instead chromatin were condensed and surrounded the nucleolus in SN-type oocytes. However, as shown in Fig. 8b, condensed chromatins were observed at not only nucleolus rim but also at various territories in the nucleus of PGC7/stella null SN-type oocytes. These results suggested that PGC7/stella is required for the condensation of heterochromatin and establishment of chromatin configuration in NSN-type oocytes and SN-type oocytes, respectively.

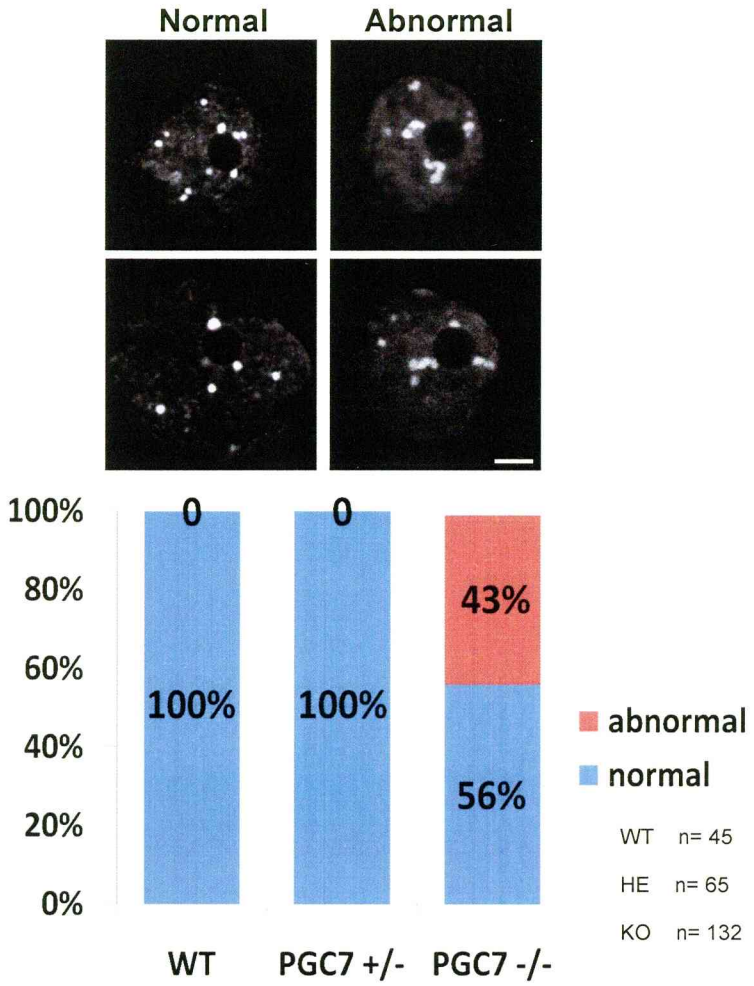


Figure 9 The heterochromatin morphology of NSN type of PGC7 null FGOs

DNA of FGOs was stained with DAPI and shown in white. The heterochromatin region which was compact and near round shape was judged as normal and which was loose and irregular shape was judged as abnormal (upper panel). The percentages of normal and abnormal NSN chromatin configuration of WT, $PGC7^{+/-}$ and $PGC7^{-/-}$ was calculated and showed in graph and indicated that all of the WT or PGC7 hetero FGO had prominent compact heterochromatin regions which were normal round shape. However, PGC7 null NSN-type FGOs had near 50% irregular shape and loose heterochromatin regions. Scale bar=10 μm

The global histone modification of PGC7/stella null FGOs

Since the global epigenetic modifications of NSN-type oocytes were different from that of SN-type oocytes (Kageyama *et al.*, 2007), I analyzed the epigenetic status of the PGC7/stella null FGOs.

SN- and NSN-type oocytes were distinguished according to the chromatin configurations deduced from images of DNA-stained, GV-stage oocytes. The modification of di- and tri- methylated lysine 9 on histone H3 (H3K9me2, H3K9me3), di- and tri- methylated lysine 4 on histone H3 (H3K4me2, H3K4me3), acetylated lysine5 on histone H4 (H4K5Ac), and acetylated lysine12 on histone H4 (H4K12Ac) were examined by immunofluorescence. As shown in Fig. 10, the levels of all the epigenetic modifications were higher in the SN type than in NSN type, these results consistent with previous report (Kageyama *et al.*, 2007). There were no significant differences in the methylation levels of H3K9me2, H3K9me3 and acetylation levels of H4K5Ac and H4K12Ac between control PGC7/stella heterozygous and PGC7/stella null FGOs (Fig. 10a, c). However the methylation levels of H3K4me2 and H3K4me3 were slightly lower in PGC7/stella null SN-type FGOs than PGC7/stella heterozygous FGOs (Fig. 10b). In addition, H3K4 trimethylation was concentrated in heterochromatin regions, but was more diffused in PGC7 KO oocyte(Fig. 10b). These data indicated that PGC7/stella is associated with several histone modifications that were acquired during the transition of NSN-type to SN-type oocytes.

Figure 10a.

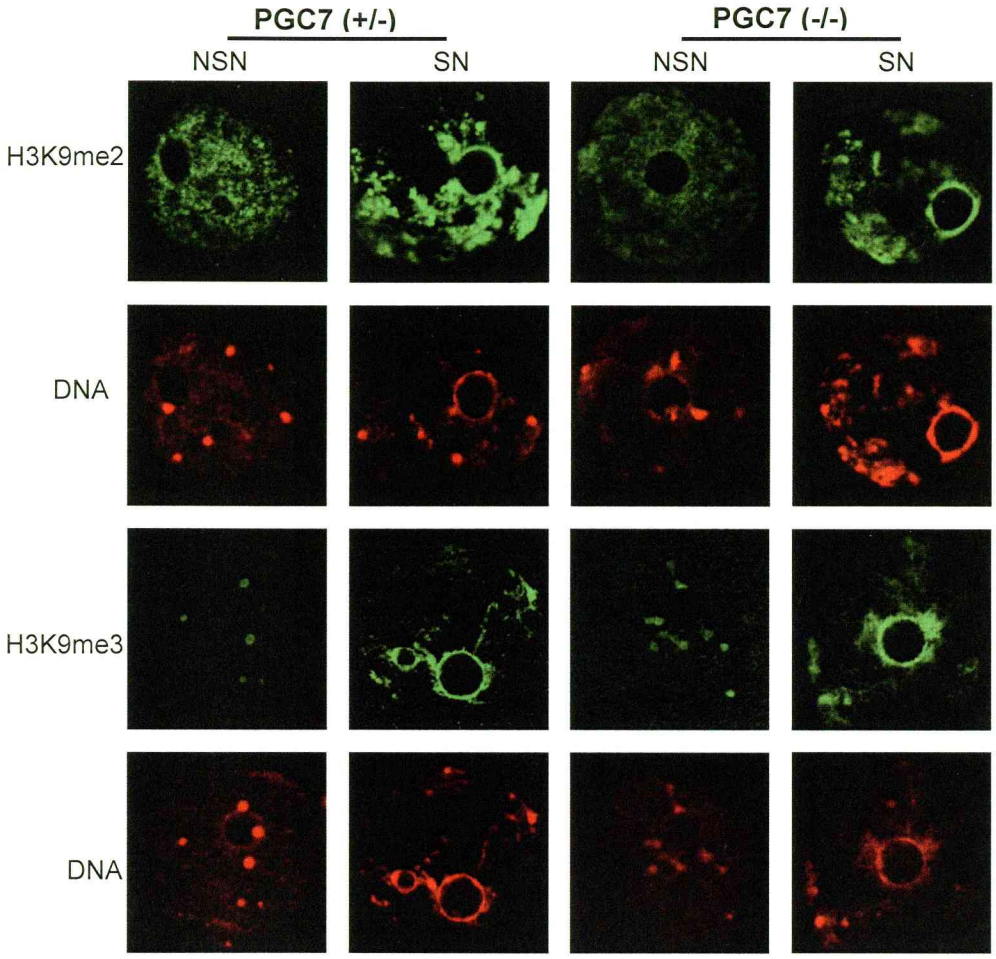


Figure 10b.

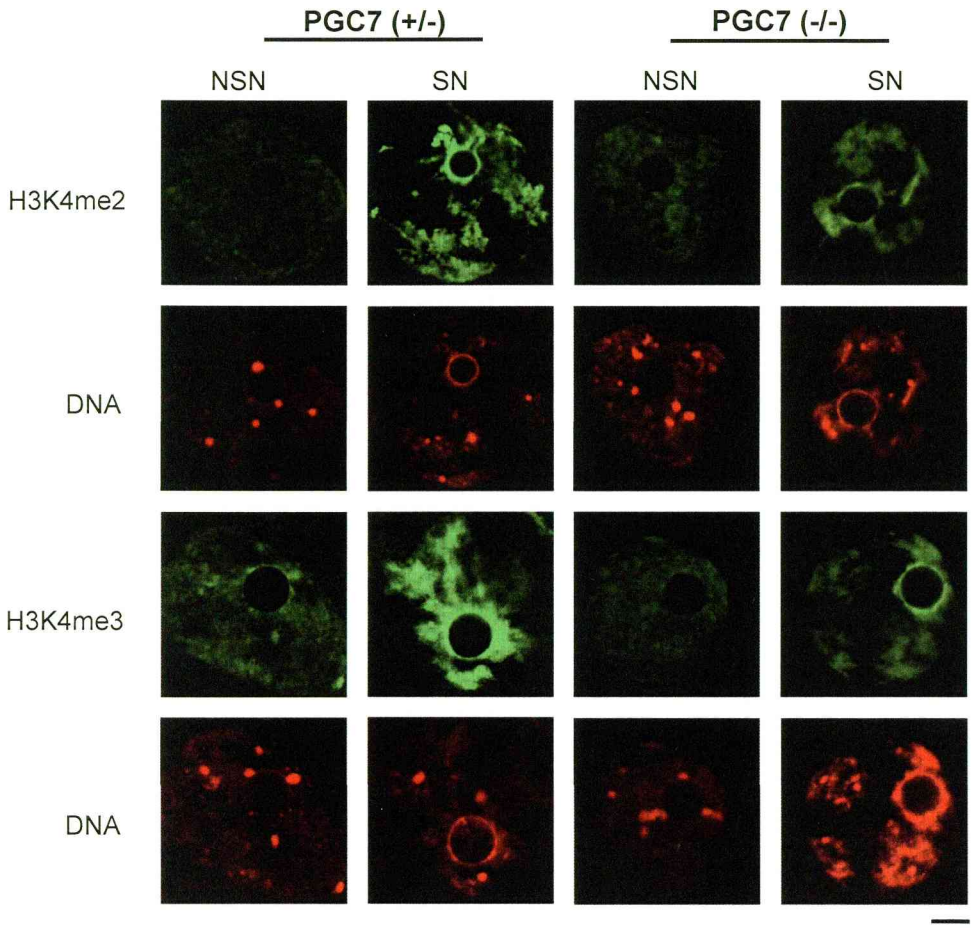


Figure 10c.

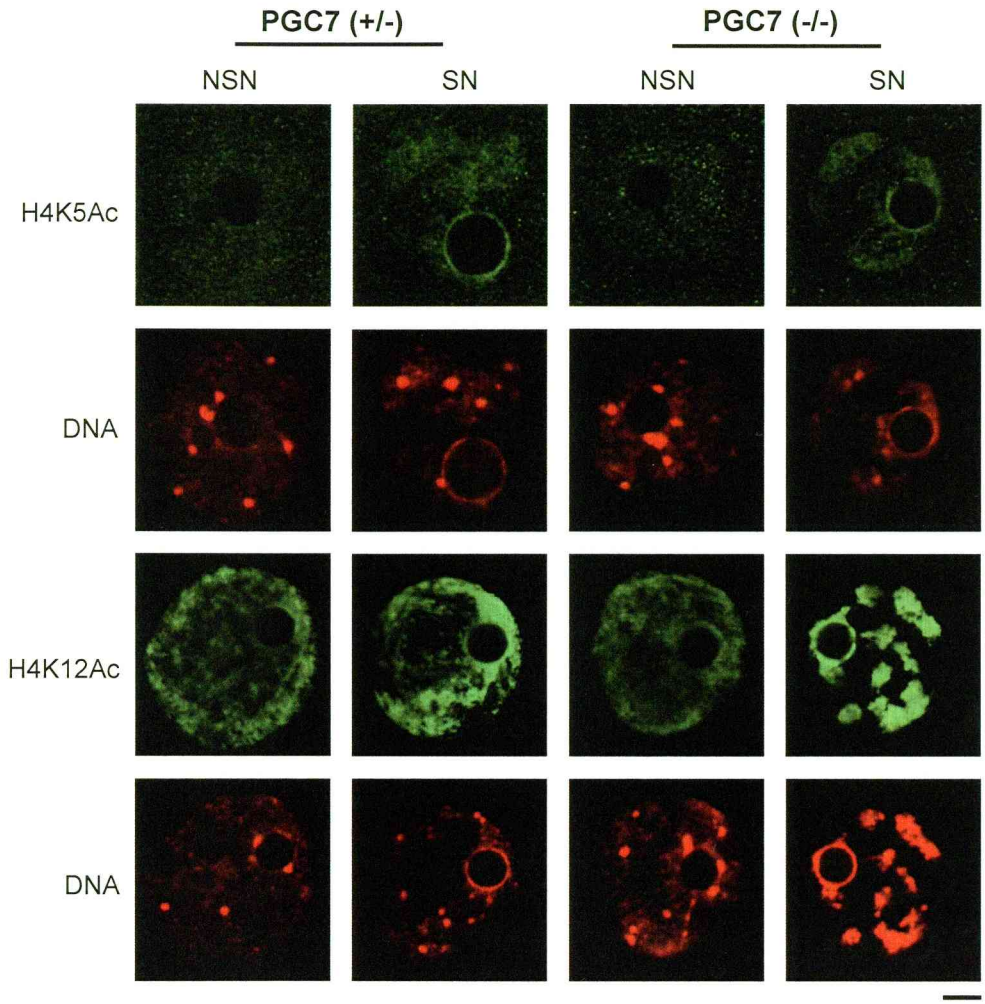


Figure 10 The global histone modification of PGC7 null FGOs

The FGOs collected from $PGC7^{+/-}$ and $PGC7^{-/-}$ female mice were immunostained with antibodies against di-methylated lysine 9 on histone 3 (H3K9me2), tri-methylated lysine 9 on histone 3 (H3K9me3) (a), di-methylated lysine 4 on histone 3 (H3K4me2), tri-methylated lysine4 on histone 3 (H3K4me3) (b), acetylated lysine 5 on histone 4 (H4K5Ac), acetylated lysine 12 on histone 4 (H4K12Ac) (c), followed by the treatment with Alexa488-conjugated anti-rabbit-Ig (H+L) antibody or Alexa488-conjugated anti-mouse-Ig (H+L), were shown in green, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and shown in red. SN- and NSN- type FGOs were distinguished according to chromatin configuration, as observed in the images of DNA stained. The levels of all the epigenetic modifications were higher in the SN type oocytes, however the modification of H3K4me2 and H3K4me3 were slightly lower in PGC7 null SN-type FGOs. Scale bar=10 μm

The transcriptional status of PGC7/stella null FGOs

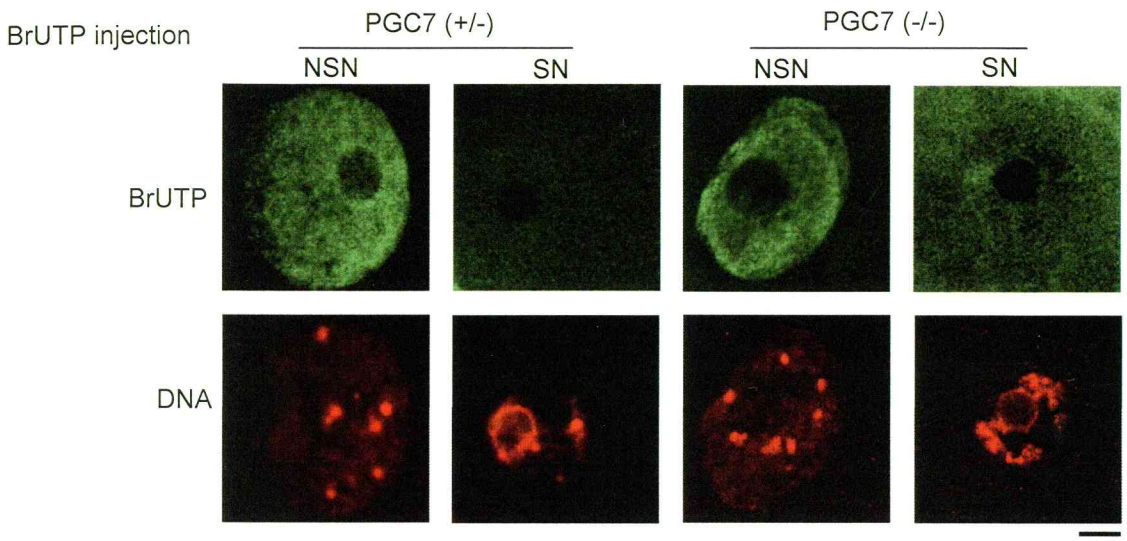
It has been reported that the transition to SN configuration is temporally coordinated with global transcriptional repression (De L Fuente and Eppig, 2001). Synthesis and storage of maternal transcripts before global transcriptional silencing enables the oocytes to complete meiosis and initiate embryogenesis. Thus, the global transcriptional repression in SN-type oocytes is a critical event for subsequent embryonic development. Since the NSN to SN transition was defected in PGC7/stella null FGOs, transcriptional activity in PGC7/stella null FGOs was determined by BrUTP incorporation. As shown in Fig. 11a, transcriptional activity was detected in either PGC7/stella heterozygous or null NSN-type FGOs. In contrast, transcription was inactive in PGC7/stella null SN-type FGOs as well as PGC7/stella heterozygous SN-type FGOs (Fig. 11a).

Consistent with the results of BrUTP incorporation, the active form of RNA polymerase II (phospho S5) was detected only in either PGC7/stella heterozygous or null NSN-type FGOs (Fig. 11b). Furthermore, activity of RNA polymerase II was decreased during the NSN to SN transition (Fig. 11b). These results indicated that transcriptional repression at SN-type oocytes normally occurred in the PGC7/stella null condition. Since the NSN to SN transition was defected in PGC7/stella null FGOs, there is a possibility that PGC7/stella is required for the global transcriptional quiescence.

Figure 11

a.

Transcription activity of PGC7 null FGOs.



b.

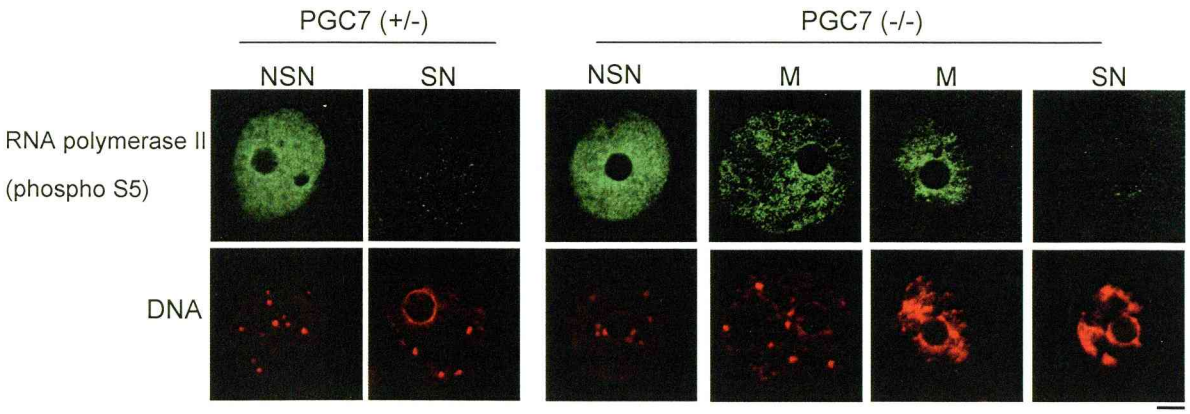


Figure 11 The transcriptional status of PGC7 null fully grown oocyte

a. Transcrptional activity was determined in FGOs derived from $PGC7^{+/-}$and $PGC7^{-/-}$ female mice. Immunofluorescent detection of BrU incorporated into nascent RNAs after microinjection of BrUTP, incubated with BrdU antibody, followed by the treatment with Alexa568-conjugated anti-mouse-Ig(H+L), were showed in green, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and shown in red. NSN chromatin configuration associated with high levels of transcriptional activity, where as SN configuration with the total cessation of all transcriptional activity.

b. $PGC7^{+/-}$and $PGC7^{-/-}$ FGOs were immunostianed with antibody against RNA polymerase II (phospho S5) to detect the active from of RNA polymerase II (phospho S5), were showed in green, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and shown in red. NSN chromatin configuration associated with high levels of RNA polymerase II activity, intermediate (M) associated with an intermediate, a slight RNA polymerase II activity, SN configuration with the almost undetectable of RNA polymerase II activity. Most of PGC7 null FGOs were NSN- and M chromatin configuration with high or slight RNA polymerase II activity. Scale bars=10 μm

Change the chromatin configuration of NSN-type FGOs to SN-type

To examine whether the PGC7/stella null NSN-type oocytes transit to SN configuration through transcription repression, PGC7/stella -null FGOs were cultured in the presence of IBMX for 1 hour. After the collection of perivitelline space lacked oocytes, which were NSN-type oocytes (Inoue *et al.,* 2007), the oocytes were cultured in the presence of 5,6-dichlororibofuransyl-benzimidazole (DRB), a potent inhibitor of transcription, for 24 hours. As shown in Fig. 12a, PGC7/stella null NSN-type oocytes could transit to SN configuration after treatment of DRB as well as PGC7/stella heterozygous NSN-type oocyte suggesting that global transcriptional repression is required for the transition of NSN to SN configuration. To determine whether the expression of PGC7/stella can rescue the phenotype of PGC7/stella null FGOs, *in vitro* transcribed PGC7/stella mRNA was miciroinjected into NSN-type PGC7/stella null FGOs and cultured for 24 hours. As shown in Fig. 12b, 25% of NSN-type PGC7/stella null FGOs transited to SN-type oocytes after PGC7/stella expression. Taken together, PGC7/stella plays an important role in the transition of NSN to SN configuration through the transcriptional repression in FGOs.

Figure 12

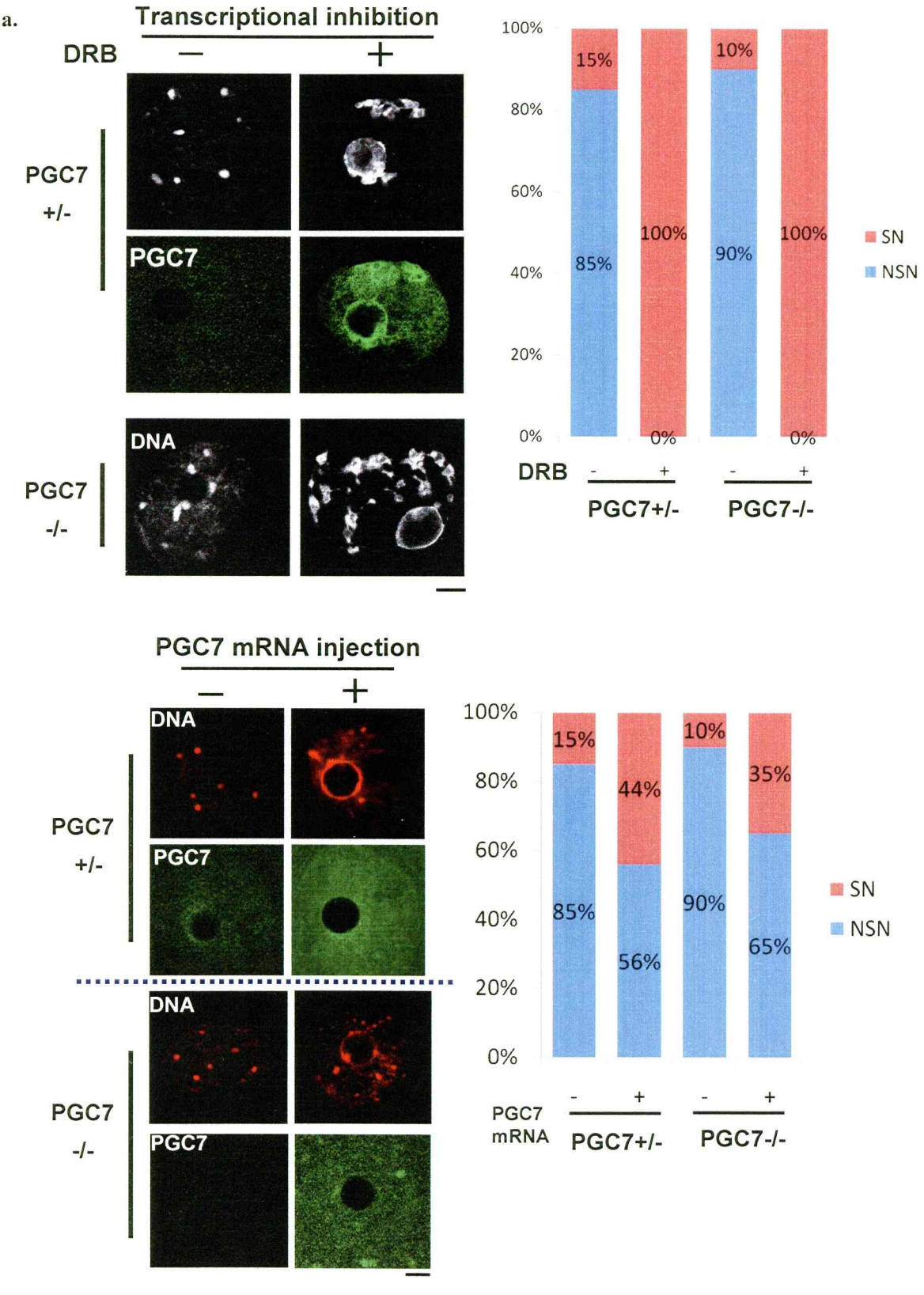

a.
Transcriptional inhibition
DRB
—
+
PGC7
+/-
PGC7
DNA
PGC7
-/-
100%
80%
60%
40%
20%
0%
15%
85%
100%
0%
10%
90%
100%
0%
SN
NSN
DRB
-
+
-
+
PGC7+/-
PGC7-/-
PGC7 mRNA injection
—
+
DNA
PGC7
+/-
PGC7
DNA
PGC7
-/-
100%
80%
60%
40%
20%
0%
15%
85%
44%
56%
10%
90%
35%
65%
SN
NSN
PGC7
mRNA
-
+
-
+
PGC7+/-
PGC7-/-

Figure 12 Change the chromatin configuration of NSN-type FGOs to SN-type

a. $PGC7^{+/-}$ and $PGC7^{-/-}$ FGOs were incubated in the presence or absent of 120 μM 5,6-dichlororibofuranosyl-benzimidazole (DRB), a transcriptional inhibitor, for 24 hours , then stained with anti-PGC7 antibody and shown in green. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and shown in white in $PGC7^{-/-}$ FGOs and red in $PGC7^{+/-}$ FGOs. All of the NSN-type FGOs became SN-type and PGC7 was concentrated around nucleolus and adjacent to heterochromatin region.

b. NSN- FGOs was collected from $PGC7^{-/-}$ female mice and injected with PGC7 mRNA then cultured for 24 hours. PGC7 protein expression was detected by immunostaining used anti-PGC7 antibody and shown in green. DNA was stained with DAPI and showed in red. NSN-type PGC7 null FGOs partially transit to SN configuration. Scale bar=10 μm

Supplement

Localization of the heterochromatin associated proteins, HP1β protein and mcroaH2A in PGC7/stella null FGOs

Since PGC7/stella is required for condensation of heterochromatin and establishment of chromatin configuration in NSN-type oocytes and SN-type oocytes, respectively. I investigated the localization of heterochromatin protein 1 (HP1 or CBX5), which is a maker of pericentric heterochromatin and associates with methylated H3K9 to form heterochromatin. And I also examined the localization of and macroH2A which is a variant of histone H2A, associated with X-chromosome inactivation and gene repression (Costanzi and Pehrson, 1998). Previous studies also show that macroH2A often resides in close proximity to percentric heterochromatin, and juxtaposition to heterochromatin protein 1β localized in heterochromatin (Hoyer-Fender *et al.*, 2004; Turner et al., 2002).

HP1β was detected in all types of fully-grown oocytes. It was localized in the regions of highly condensed chromatin. All NSN oocytes had randomly distributed regions of highly condensed heterochromatin, (Fig. 13arrows). Such regions, but less numerous were also observed in the majority of SN oocytes (Fig. 13 arrows). In some of the SN oocytes, the highly condensed chromatin regions were absent. HP1β was also found on some fragments of chromatin "rim" surrounding the nucleolus in SN oocytes (Fig. 13, arrowhead). In these fragments, chromatin was more condensed than in the other parts of chromatin "rim". These results were consistent with previous report (Meglicki *et al.*, 2008). However, compared with the PGC7/stella heterozygous, PGC7/stella null FGOs had more diffused heterochromatin regions with irregular shape (Fig. 13). Although the expression level is not different form control, the observation of heterochromatin shape

is quite irregular in both NSN- and SN-type of PGC7/stella null oocytes

The similar result also can be observed in the immunostaining of macroH2A. MacroH2A was concentrated in the condensed heterochromatin regions of NSN or SN type oocyte. In addition, irregular shape and less condensed heterochromatin regions were localized with macroH2A in PGC7/stella null FGOs.

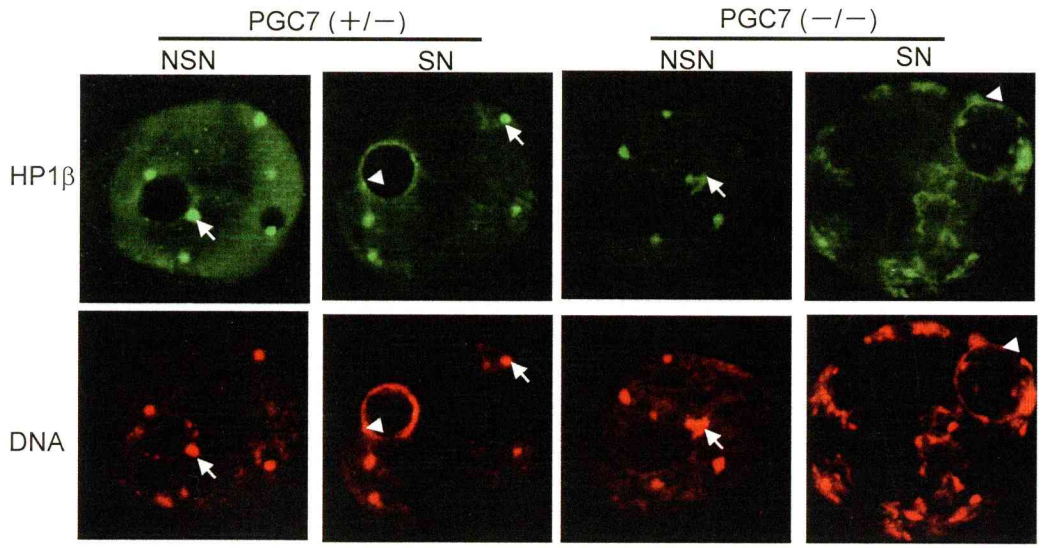


Figure 13 Localization of HP1b Proteins in PGC7/stella null FGOs

The FGOs collected from PGC7$^{+/-}$ and PGC7$^{-/-}$ female mice were immunostained with antibodies against heterochromatin protein β1 (HP β1), followed by the treatment with Alexa488-conjugated anti-mouse-Ig (H+L) antibody were shown in green. The Chromatin stained with DAPI shown in red, arrows indicate the regions of highly condensed chromatin, arrowheads indicate most condensed fragments of the chromatin ''rim,'' stars indicate chromatin-free area in the nucleoplasm. In NSN oocytes, HP1b protein is present in the heterochromatin domains (arrows), in the nucleoplasm, and in highly condensed fragments of chromatin rim surrounding the nucleolus in SN oocytes. Although the expression level is not different form control, however the observation of heterochromatin shape is quite irregular in PGC7/stella null both NSN and SN oocytes.

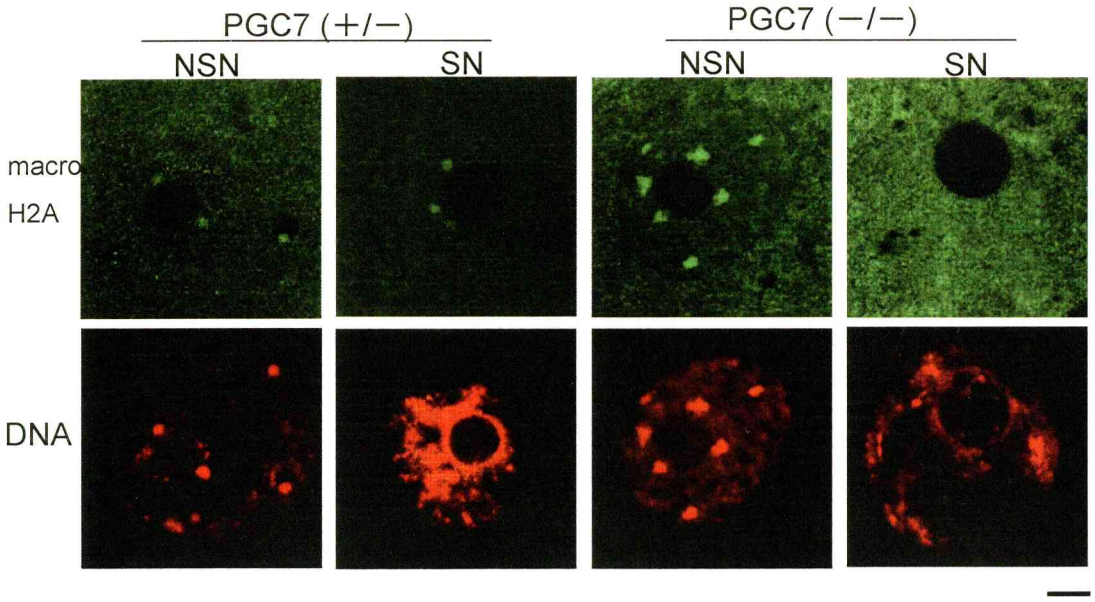


Figure 14 Localization of macroH2A in PGC7/stella null FGOs

The FGOs collected from PGC7$^{+/-}$ and PGC7$^{-/-}$ female mice were immunostained with antibodies against macroH2A, followed by the treatment with Alexa568-conjugated anti-rabbit-Ig (H+L) antibody were shown in green. The Chromatin stained with DAPI shown in red. MacroH2A protein was restricted to condensed chromatin region in both NSN and SN oocytes.

Bar = 10μm

Discussion

Alteration of chromatin structure and functions in FGOs is an important epigenetic mechanism for the control of gene expression during oocyte maturation. However, the cellular and molecular mechanisms coordinating dynamic change in large-scale chromatin structure with the onset of transcriptional repression are poorly understood. In this study, I examined the function of PGC7/stella, a maternal effect gene required for the early embryonic development, during the oocyte maturation. First, I found that the localization of PGC7/stella were different in NSN- and SN-type FGOs. Although the localization of PGC7/stella was diffusely localized in NSN-type oocyte, its localization was progressively concentrated around the nucleolus in SN-type oocytes (Fig. 7). I also found that transition of NSN to SN-type during oocyte growth was defected in PGC7/stella null condition (Table 1, Fig. 8a). In addition, irregular shape and less condensed heterochromatin regions were observed in PGC7/stella null FGOs (Fig. 8b, Fig. 9). Zuccoti et al reported that the expression of PGC7/stella was decreased in metaphase II (MII) oocytes originated from NSN oocytes. Since NSN-type oocytes did not possess the developmental competence, the expression of PGC7/stella during oocyte maturation might be important for the developmental competence.

It has been reported that histone modification, such as acetylation, methylation and phosphorylation, play important roles in the regulation and gene expression. Some modifications, e.g. acetylation of most of the lysine residues in histone H3 and H4 and methylation of H3K4, associated with transcriptional activation, while the other modifications, e.g. methylation of H3K9, relative to transcription repression (Jenuwein & Allis 2001, Kurdistani *et al.*, 2004, Bernstein *et al.*, 2005). However the levels of most of histone modifications were higher in SN-type oocytes than in the NSN-type

oocytes (Kageyama *et al.*, 2007). Although transcriptional activity decreased during oocyte growth (Worrad *et al.*, 1994), the extents of acetylated histones and methylation of histone H3K4, which are associated with active transcription, increased. Therefore, the globe histone modification may be involved in genome-wide alternation of chromatin modification, which is not associated with transcription. I analyzed the epigenetic modification statuses of PGC7/stella null FGOs. As well as PGC7/stella heterozygous FGOs, the levels of H3K9me2, H3K9me3, H4K5Ac and H4K12Ac were increased during oocyte growth (Fig. 10a, c). However, the levels of H3K4me2 and H3K4me3 were slightly decreased in PGC7/stella null SN-type FGOs than that in PGC7/stella heterozygous SN-type FGOs (Fig. 10b). These data demonstrated that PGC7/stella is associated with several histone modifications that were acquired during the transition of NSN-type to SN-type oocytes.

After transcriptional repression, pre-ovulatory oocytes use maternal messenger RNA (mRNA) stores to resume meiosis and sustain the first cleavage divisions after fertilization (Hodgman *et al.*, 2001; Stebbinsboaz *et al.*, 1996). Thus, the timing of transcriptional repression is critical for subsequent embryo development. In mammalian somatic cells, transcriptional repression occurs during the transit through mitosis (Gottesfeld and Forbs, 1997). However, global transcriptional repression in the FGOs occurs long before germinal vesicle breakdown and the condensation of individual chromosomes. In contrast to somatic cells, unique strategies are set in place for the control of transcriptional silencing in pre-ovulatory oocytes (De La Fuente *et al.*, 2004). I examined the transcriptional status of PGC7/stella null oocytes by BrUTP incorporation. My results revealed that PGC7/stella null NSN-type oocytes exhibit high transcriptional activity and as well as control NSN-type oocytes (Fig. 11a). In contrast,

PGC7/stella null SN-type oocytes lacked transcriptional activity similar to that in control SN-type oocytes. I also performed immunofluorescence to detect the active form of RNA polymerase II. Consistent with the results of BrUTP incorporation, the RNA polymerase II has much higher activity in NSN-type oocytes, and then gradually decreased in the intermediate type oocytes, finally almost undetectable in SN-type oocytes (Fig. 11b). These results demonstrated that transcriptional repression at SN-type oocytes normally occurred in the PGC7/stella null condition. However, there is a possibility that PGC7/stella plays some role in transcriptional repression because most of PGC7/stella null FGOs were arrested NSN-type oocytes stage.

Chromatin condensation around the nucleolus is associated with global transcriptional silencing in pre-ovulatory oocytes. However, the analysis of FGOs lacking nucleoplasmin 2 (Npm2), in which transition to SN-type configuration was severely compromised, revealed that transcriptional silencing occurs in mutant oocytes despite the absence of chromatin remodeling to SN-type configuration (De La Fuente 2004). Thus, chromatin remodeling into SN-configuration and transcriptional repression can be dissociated and are regulated through different pathways. In contrast, I demonstrated that the treatment of transcriptional inhibitor induced the transition of NSN- to SN-type chromatin configuration even in the PGC7/stella null condition (Fig. 11a). This data strongly indicated that transcriptional repression is prerequisite for the transition of NSN to SN configuration. Finally, I attempted to induce the chromatin configuration from NSN to SN by introduced the PGC7/stella mRNA to PGC7/stella null NSN oocytes. My result indicated that some NSN-type PGC7/stella null oocytes transited to SN-type oocytes. Taken together, PGC7/stella is required for the transition of NSN- to SN-chromatin configuration may be though the transcriptional repression

during the oocytes maturation. I propose that PGC7/stella has novel functions in oocytes before fertilization and affect the acquisition of developmental competence.

The role of PGC7/stella during large scale chromatin remodeling

It has been reported that the heterochromatin surrounding the nucleolus in SN oocytes is mainly AT-rich satellite-DNA of centromeric origin and involves most of the centromeres, whereas in NSN oocytes only the centromeres carrying the nucleolar organizing regions (NORs) are associated with the nucleolus (Fig. 15)(Longo *et al.*, 2003). However, in the nucleus of PGC7/stella null SN-type oocyte condensed chromatins were observed at not only nucleolus rim but also at various territories. Thus, PGC7/stella may play a role for DNA organization lead the most of heterochromatin can condense around the nucleolus.

Figure 15

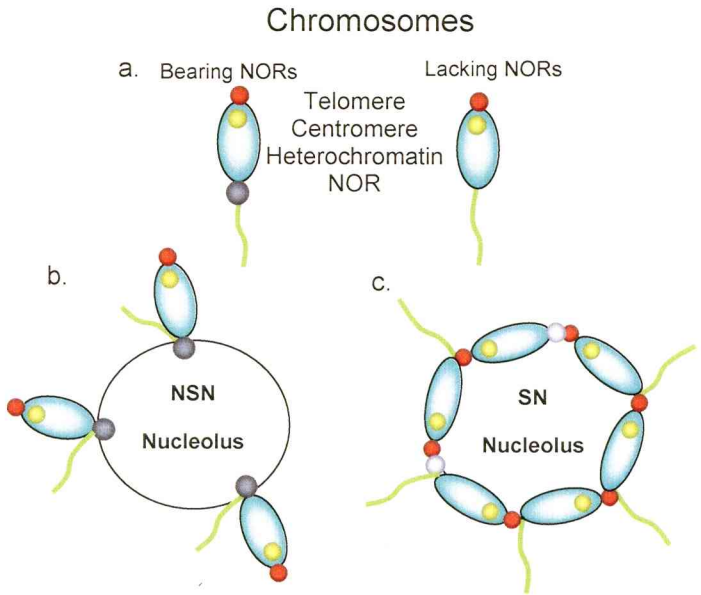


Figure 15 Proposed model depicting how chromosomes possessing and not possessing NORs might be associated with the nucleolar surface of NSN and SN oocytes

a: Schematic representation of the pericentromeric region of chromosomes bearing and not bearing NORs. Model of the nuclear architecture of NSN (b) and SN (c) oocytes: In both types of oocytes, NOR-bearing chromosomes are always associated with the nucleolus; in NSN oocytes they are transcriptionally active (grey sphere), whereas in SN oocytes they become inactive (white sphere). The heterochromatic regions of chromosomes not possessing NORs are dispersed within the nucleoplasm of NSN oocytes (b); but they cluster around the nucleolar surface in SN oocytes (c), contributing to the formation of a Hoechst-positive rim that might be involved in the repression of gene expression at this stage of cytodifferentiation which precedes ovulation.

The function of the maternal effect gene, PGC7/stella during oogenesis

A mammalian oocyte is the only known cell that can activate a zygotic genome after fertilization and reprogram a somatic nucleus into pluripotent state. Therefore, several genes specifically expressed in oocytes are likely responsible for the ability to reprogram genomes as well as for oogenesis. Oocyte specific transcription factor such as *Figla* and *Nobox* are expression througout folliculogenesis and most like play a critical function in accumulation of transcripts necessary for oocyte growth and early embryogenesis. Maternal effect genes such as HSF1, MATER, NPM2, PGC7/stella and ZAR1 are all required for the normal embryonic development beyond the one-cell or two-cell stage (Table2 and Fig. 16). However, the functions of these maternal effect genes during oogenesis are still unclear.

The SN chromatin configuration of FGO which is transcriptional inactive and associated with developmental competence, however the mechanism of SN configuration formation remains unclear. Recent report indicated that both the properties in nucleus and cytoplasm of SN-type FGO are required for early embryonic genesis (Inoue *et al.*, 2007). Thus, several maternal effect genes may involve in the SN configuration formation in FGO. It has been reported that NPM2 which is important for nucleoli formation during oogenesis has no SN type FGOs in NPM2 knockout mice (De La Fuente *et al.*, 2004). In the present study, I found that PGC7/stella null mouse has reduced SN-type oocytes in FGOs. Thus, PGC7/stella may play a role for SN-chromatin formation and involve in the global transcriptional repression. Since PGC7/stella encodes for a basic protein with a SAP like domain thought to have a role in chromatin organization. In addition, PGC7/stella has a strong DNA binding ability that may recruit transcriptional repressor to chromatin to induce transcriptional cessation. There are still

many oocyte specific transcripts which are functional unknown. I should identify the factors that associated with PGC7/stella that may play a function in the SN configuration formation and transcriptional inactivation in FGO in the future.

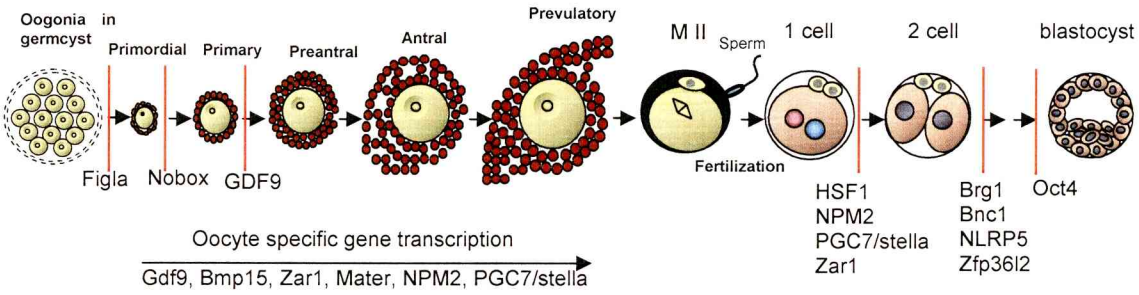


Figure 16 Knockout mouse phenotypes of genes preferentially expressed in oocytes

Knockout mouse phenotype of transcriptional factors preferentially expressed in oocytes such as Figla, Nobox, and GDF9 is loss oocyte or folliculogenesis arrest at early stage of postnatal development. Knockout mouse phenotype of maternal effect gene: maternal mRNAs that are used to direct embryonic development before zygotic genome activation, such as Brg1, Bnc1, NLRP5, HSF1, NPM2, PGC7/stella, ZAR1 and ZFP36l2 is embryo arrest at 1 cell or 2cell and rarely reach to blastcyst stage.

Table2 Knockout mouse phenotypes of genes preferentially expressed in oocytes

Gene	Name	Mouse knockout phenotype	References
Transcription factor, preferentially expressed during oogenesis			
Figla	Factor in the germline alpha	Infertility; oocyte loss by postnatal day 2	Soyal *et al.*, (2000)
Nobox (*Og2x*)	Newborn ovary	Infertility; oocyte loss by postnatal day 14; disrupted primordial to primary transition	Rajkovic *et al.*, (2004)
Oct4 (*Pou5f1*)	POU domain, class 5, transcription factor 1	Maintenance of primordial germ cells	Kehler *et al.*, (2004)
Gdf9	Growth differentiation factor-9	Infertility; folliculogenesis arrest at the one-layer follicle stage	Dong *et al.*, (1996) Elvin *et al.*, (1999)
Nr6a1 (*Gcnf*)	Germ cell nuclear factor	Subfertility; prolonged diestrus	Lan *et al.*, (2003)
Taf4b (*TAFII105*)	TATA box binding protein-associated factor 4b	Infertility; folliculogenesis blocked at pre-antral stage	Freiman *et al.*, (2001)
Maternal effect gene			
Brg1	brahma (Brm) or brahma-related gene 1	Infertility; development beyond the two-cell stage is blocked	Bultman *et al.*, (2006)
Bnc1	Basonuclin 1	Subfertility; eggs fail to develop beyond the two-cell stage	Jun *et al.*, (2006)
Dnmt1	DNA methyltransferase 1	Subfertile; embryos of knockout females die during gestation due to imprinting defects	Howell *et al.*, (2001)
Hsf1	Heat shock factor 1	Infertile; pre-and post-implantation defect	Xiao *et al.*, (1999) Christians et al., (2000)
NLRP5(Mater)	NLR family, pyrin domain containing 5	Infertile; fertilized embryo offspring do not progress beyond the 2-Cell stage	Tong *et al.*, (2000)
Npm2	Nucelophosmin 2	Subfertile;partial block at 1 cell to 2 cell embryo stage	Burns *et al.*, (2003)
Pgc7 (*stella;DPPA3*)	Primordial germ cell 7	Infertile; defective preimprintation embryonic development	Payer *et al.*, (2003) Nakamura *et al.*,(2007)
Ube2A, (HR6A)	Ubiquitin-conjugating enzyme E2A, RAD6 homolog	Infertile; fertilized embryo offspring do not progress beyond the 2-Cell stage	Roest et al., (2004)
Zar1	Zygote arrest1	Infertile; block at 1 cell to 2cell embryo stage	Wu *et al.*, (2003)
Zfp36l2	Zinc finger protein 36 C3H type-like 2	Infertile; embryo arrest at 2 cell stage	Ramos *et al.*, (2004)
Zfp57	Zinc finger protein 36	Embryonic and neonatal lethality	Li *et al.*, (2008)

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Achievement

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