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DNA Methylation, Histone Modifications and Behaviour of AKAP95 during Mouse Oocyte Growth and Upon Nuclear Transfer of Foreign Chromatin into Fully Grown Prophase Oocytes*

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The poor efficiency of mammalian cloning is due to inappropriate/incomplete epigenetic reprogramming of the donor chromatin. As the success in reprogramming of the donor nucleus may require activity of similar mechanisms which reprogram the chromatin in the course of gametogenesis, we decided to follow the status of some epigenetic markers in the late phase of oogenesis in mice, i.e. in prophase oocytes during their growth and after completion of the growth phase. Our analysis reveals an increase in the level of global DNA methylation starting in oocytes with diameters around 60μm which was further elevated until completion of oocyte growth. A similar increase was observed in respect to the acetylation of histone H4. On the other hand, the methylation of histone H4 Arg3 was constantly high until the end of oocyte growth, although it differed between fully grown oocytes depending on the type of spatial chromatin organization. We have also studied the AKAP95 protein which was abundant at earlier stages but decreased in fully grown oocytes according to changes in their chromatin organization. The nuclear transfer of different types of donor nuclei with hypomethylated DNA into fully grown prophase oocytes did not increase the global level of methylation of transferred foreign chromatin, regardless if the recipient oocyte was devoid of its own nucleus or its nucleus was left intact. This suggests a major problem in the ability of recipient oocytes to modify donor DNA methylation.

Key words: Mouse oocyte, chromatin, DNA methylation, histone status, AKAP95, nuclear transfer.

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The creation of a new individual comprises a paradox: on the one hand, oocyte and sperm are highly differentiated cells, specialized to fulfill a unique function, on the other hand their chromatin possesses the potential to execute the full developmental program upon joining at fertilization. This totipotency acquired during gametogenesis is then gradually lost as the embryo develops due to silencing of genes the expression of which is no longer required in cells that have passed a specific developmental stage (RIDEOUT *et al*. 2001). The only exceptions to this rule are cells of the germ line in which chromatin is reprogrammed to regain full developmental competence.

In the mouse it was shown that the critical stage at which oocyte chromatin acquires the ability to support full embryonic development corresponds to the late phase (starting in oocytes with a diameter around 50-60 μm) of oocyte growth (BAO *et al*. 2000). It is commonly accepted that reprogramming of oocyte chromatin involves a set of epigenetic modifications. Examination of the pattern of DNA methylation in specific sequences indeed proved that genomic imprinting, a process contrib-

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uting to reprogramming, takes place during oocyte growth (OBATA & KONO 2002; HIURA *et al.* 2006). Other study reported that methylation and acetylation of histones as well as the level of global DNA methylation changes during oocyte growth in mice (KAGEYAMA *et al.* 2007). The results of this last report (KAGEYAMA *et al.* 2007), however, were obtained in prepubertal mice from oocytes of the first wave of growth. Since such oocytes do not ovulate, it is important to determine whether similar histone modifications occur also in adult females, i.e. in growing oocytes which subsequently undergo ovulation and fertilization.

Growing oocytes possess a specific type of spatial organisation of the nucleus, called a non-surrounded nucleolus (NSN) with loosely condensed, evenly distributed chromatin. This organisation, however, changes at the end of the growth phase. Accordingly, among fully grown oocytes competent to resume meiotic maturation, some still exhibit the NSN organisation of chromatin. In other fully grown oocytes, chromatin develpos the so-called surrounded nucleolus (SN) organisation in which chromatin is much condensed and localized mainly around the nucleolus (DEBEY *et al*. 1993; ZUCCOTTI *et al.* 1995). These two states of chromatin organization differ functionally because SN oocytes develop with high efficiency following fertilization, whereas the development of fertilized NSN oocytes is impaired (ZUCCOTTI *et al.* 2002).

The full developmental potential of chromatin of the gametes is gradually lost after fertilization as the embryo develops (HIIRAGI & SOLTER 2005) due to gradual transcriptional silencing of developmentally important genes. This is probably the major obstacle in the wide implementation of somatic cell nuclear transfer (SCNT) and related technologies. The poor outcome of SCNT seems to reflect the epigenetic status of differentiated donor nuclei which differ from the state of the reprogrammed chromatin in the functional gamete (reviewed by RIDEOUT *et al*. 2001; EGLI *et al*. 2008). Successful cloning therefore requires reprogramming of the donor chromatin in the recipient oocyte cytoplasm (HEYMAN *et al*. 2002; HIIRAGI & SOLTER 2005). This raises the question of whether reprogramming of the germ cell chromatin as well as successful reprogramming of the donor nucleus upon nuclear transfer may rely on similar epigenetic mechanisms. In both cases, however, these mechanisms remain obscure.

In this study we have carefully examined changes in epigenetic markers during oocyte growth in adult mice. We have found that the distinct increase in global DNA methylation in oocytes occurs in a later stage of oocyte growth, thus coinciding with the timing of acquisition of the full developmental potency of the oocyte chromatin reported previously (BAO *et al*. 2000). Our nuclear transfer experiments demonstrate, however, that the cytoplasm of fully grown oocytes is unable to change the global DNA methylation status of transplanted undermethylated donor nuclei. We show that compared to growing oocytes in prepubertal mice (KAGEYAMA *et al.* 2007), the epigenetic status of chromatin changes in a similar way in the growing oocytes of sexually mature females. We also show that the transition from NSN to SN type of oocyte chromatin is associated with dynamic changes in the level of the nuclear matrix protein AKAP95.

Material and Methods

Mice, oocytes and fertilized eggs

Mice from an outbred line OF1 were used throughout the experiments. The experiments were approved by the Local Ethics Committee No. 1 in Kraków (resolution number 53/2008 of 10 July 2008), Poland and Regierungspraesidium in Freiburg, Germany, according to European Union Council Directive 86/609/EEC of 24 November 1986.

The dissected ovaries were placed in M2 culture medium and pricked intensively to release the fully grown oocytes as well as the preantral follicles. Fully grown oocytes at the GV stage were collected and transferred into M2 medium supplemented with dbcAMP (150 μg/ml, Sigma) to prevent resumption of meiosis. Preantral follicles of different size were collected and then treated with trypsin (1.5 mg/ml in M2, Sigma) for 25 minutes at 37^{o}C. Following enzymatic treatment the suspension was vigorously pipetted and then the released growing oocytes of different sizes were collected. Immediately after collection the oocyte diameter was measured and the oocytes were allocated into different size groups.

To obtain fertilized eggs the females were superovulated and mated as described previously (POLANSKI *et al*. 2005). Zygotes at the G2 stage were collected 28 hours after hCG injection and served as the source of pronuclei for pronuclear transfer. At this stage the DNA in the paternal pronucleus is already highly demethylated, in contrast to strongly methylated DNA in the maternal pronucleus (MAYER *et al.* 2000).

Nuclear transfer and culture of micromanipulated oocytes

The transfer of the pronucleus from fertilized eggs or the germinal vesicle from growing oocytes into fully grown oocytes was performed as de-

scribed previously (POLANSKI *et al.* 2005). The paternal and maternal pronuclei were distinguished on the grounds of their distance from the second polar body: the male pronucleus is much more distant than the female pronuclei. The minority of the zygotes in which the pronuclei did not differ clearly in their distance from the second polar body were not used as donors. After fusion of the karyoplasts with the cytoplasm of the recipient fully grown oocytes, the resulting oocytes were rinsed and then placed into dbcAMP-free medium to allow resumption of meiotic maturation. Depending on the experiment, the micromanipulated oocytes were cultured in this medium for 6.5 or 18 hours reaching the stage equivalent to metaphase I or metaphase II, respectively.

Immunofluorescence

For DNA methylation the oocytes were processed in order to prepare the chromatin as described by TARKOWSKI (1966). The oocytes were then stained using the primary antibody recognizing 5-methylcytosine (Eurogentec) and the FITC conjugated goat antimouse secondary antibody, followed by DAPI as described (POLANSKI *et. al* 2008). However, in preliminary experiments we were unable to detect the signal both from the secondary antibody as well as from DAPI on preparations from GV stage oocytes (growing as well as fully grown). Most likely, the lack of a detectable signal resulted from the high level of decondensation of the chromatin in GV oocytes. To overcome this problem, we first cultured the GV oocytes for 24 hours in M2 supplemented with okadaic acid (2.5 μM, Sigma) which induces the condensation of chromatin in GV oocytes including growing ones which are incompetent to resume meiotic maturation (ALEXANDRE *et al*. 1991; GAVIN *et al*. 1991; POLANSKI 1997). After culture the oocytes were fixed using the TARKOWSKI technique (1966) and stained as described above.

For histone modifications the oocytes were fixed for 30 minutes in 2.5% paraformaldehyde supplemented with 0.1% triton X, following 30 min incubation in 2% Triton. After rinsing the oocytes were blocked for 30 minutes in 3% BSA and incubated overnight with the primary antibody at 4^{o}C. Following rinsing the oocytes were incubated with the secondary antibody for 1 h at room temperature, rinsed and stained with DAPI (2 μg/ml, 4 minutes). The primary antibodies were rabbit anti-acetyl histone H4 (recognizing acetylation at lysines 5, 8, 12 and 16) and rabbit anti-dimethyl-histone H4Arg3 (both from Upstate), both used at a concentration of 1:200. As the secondary antibody, in both cases, we used goat anti-rabbit FITC conjugated antibody (Santa Cruz Biotechnology) at a concentration of 1:100.

To detect AKAP95 we applied rabbit anti-AKAP95 antibody (Upstate,Millipore) at a concentration of 1:250 using the same protocol as for the detection of histone modifications except that the oocytes were blocked in 10% goat serum. Goat anti-rabbit FITC conjugated antibody (Santa Cruz Biotechnology) at a concentration of 1:100 was used as the secondary antibody.

All sollutions used for fixation and staining were made in PBS. The preparations were mounted in Citifluor.

Quantification of DNA methylation, histone modifications and AKAP95 were performed by measuring the fluorochrome signal using Image J software. For each preparation the values obtained for growing oocytes were normalized to the mean value of the signal measured in fully grown oocytes on the same preparation.

Results

We have followed three epigenetic markers in different size categories of mouse prophase oocytes. In all three cases the analysis of variance revealed a significant change in chromatin status (Fig. 1ABC). The global methylation of DNA started to increase significantly in oocytes which reached around 60 μm in diameter and then the level of methylation was elevated further until the end of the growth phase. Histone H4 acetylation exhibited a similar profile although the start of the increase occurred at a later phase of oocyte growth. On the other hand, the level of histone H4 Arg3 methylation was stable and decreased slightly, although significantly, only in fully grown oocytes which attained the SN-type configuration of chromatin.

We analyzed AKAP95 which was shown previously to participate in the changes of chromatin organization (WANG *et al*. 2001) to examine whether epigenetic modifications in prophase oocytes may accompany the changes in the expression/localization of this protein. We observed an abundance of AKAP95 until the end of oocyte growth, followed by a dramatic drop in the fully grown oocytes undergoing major changes in chromatin conformation from NSN to SN-type (Fig. 2).

To check whether the fully grown prophase stage oocyte is able to change the epigenetic status of the foreign chromatin, we performed nuclear transfer of different types of the donor nuclei: the hypomethylated male pronucleus and hypermethylated female pronucleus (control) both from the G2 zygotes as well as the hypomethylated pro-

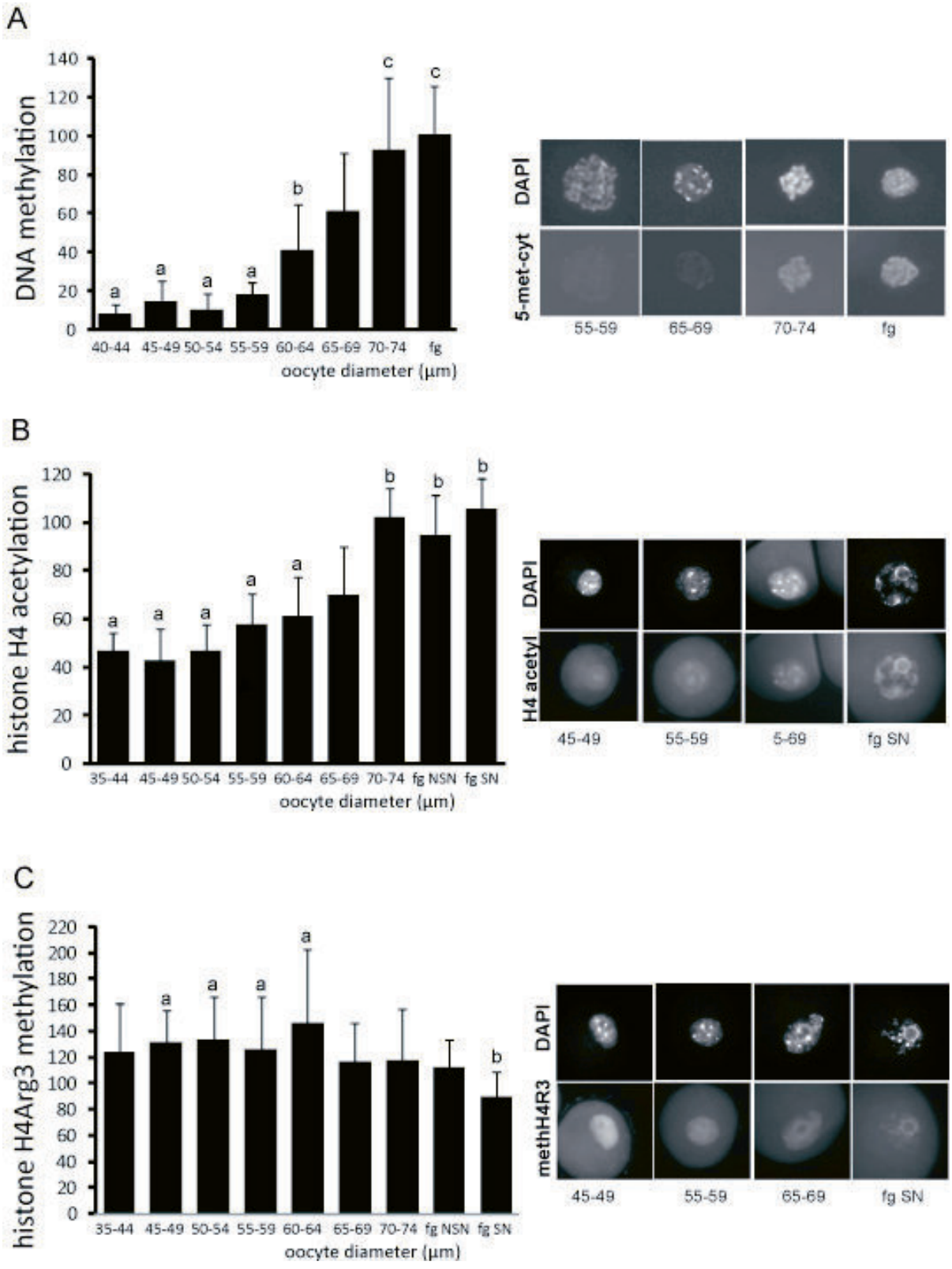

Fig. 1. Epigenetic status of chromatin in different size categories of growing mouse oocytes. A – DNA methylation; B – Histone H4 acetylation; C – Histone H4Arg 3 methylation. Data represent mean ± SEM. Typical examples are shown on the right. Different letters indicate statistically significant differences (Kruskal-Wallis ANOVA on ranks with Dunn's all pairwise multiple comparison *post hoc* tests; $p<0.05$).

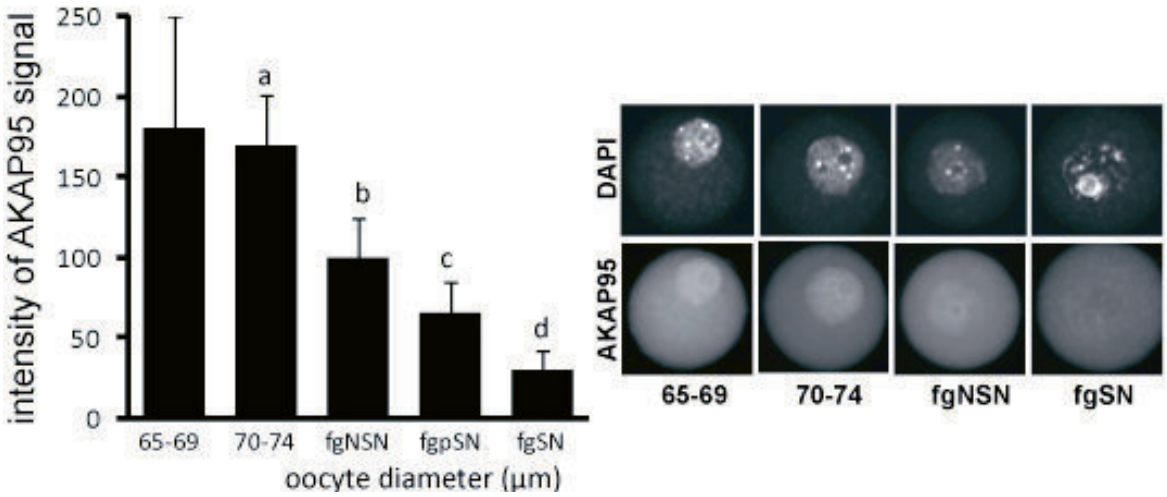

Fig. 2. Changes in the level of AKAP 95 at the end of the growth phase in mouse oocytes. Left – the mean values (±SEM) for different categories of oocytes; right – typical examples. Different letters indicate statistically significant differences (Kruskal-Wallis ANOVA on ranks with Dunn's all pairwise multiple comparison *post hoc* tests; $p<0.05$).

phase (GV-stage) nucleus from growing oocytes (40-49 μm diameter). For the pronuclear transfer the recipient prophase oocyte was first enucleated and after pronucleus transplantation cultured for 18 hours. In case of GV transfer, the recipient oocyte was intact (not enucleated) and the culture after GV transfer was performed for 6.5 hours. In both cases in which hypomethylated donor nuclei were transferred (male pronucleus or GV from growing oocytes) we did not observe any significant increase in the global methylation of their DNA after transfer and culture (Table 1, Fig. 3). The GV transfer into intact recipients enabled the direct comparison between the states of DNA methylation of the recipient oocyte and donor nucleus in the same cytoplasm. In all cases of such transfer, half of the condensed bivalent chromosomes revealed high methylation (typical for fully grown oocytes), whereas the other half (obviously representing the bivalents resulting from condensation of donor chromatin) remained hypomethylated (Fig. 3).

Table 1

The level of DNA methylation after nuclear transfer of different donor nuclei into fully grown prophase oocytes

Methylation of donor chromatin	Number of donor nuclei		
	female PN (control)[a]	male PN[a]	GV from 40-49 μm oocyte[b]
low	0	16	7
medium	1	9	0
high	19[#S]	1[#]	0[$]

[a]Transfer into enucleated recipient with subsequent culture for 18 hours.

[b]Transfer into intact (not enucleated) recipient with subsequent culture for 6.5 hours.

#the proportions of the donor nuclei with high level of DNA methylation differ significantly ($p<0.001$; chi square 2X2 table with Yates correction).

[$] the proportions of the donor nuclei with high level of DNA methylation differ significantly ($p<0.001$; Fisher exact test).

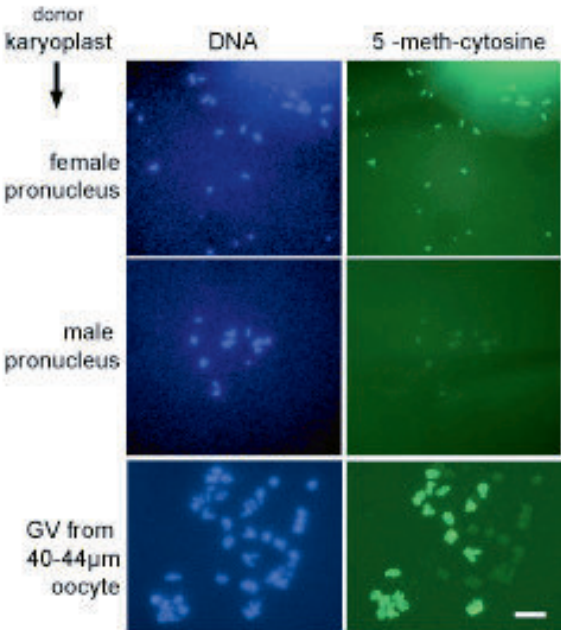

Fig. 3. Typical examples of DNA methylation of different donor nuclei after nuclear transfer into fully grown prophase oocytes. Upper and middle row – transfer into enucleated recipient; lower row – transfer into intact recipient. Note that in the case of GV transfer exactly 20 bivalents reveal high methylation and another 20 are hypomethylated. Scale bar – 20 μm.

Discussion

Since the first successful mammalian cloning (WILMUT *et al*. 1997), wide implementation of this technology in farm/domestic animals is still hampered due to the low efficiency of this process. The frequency of obtaining live-born cloned pups differs between species, nevertheless the overall rate of development to live offspring remains on the order of 1-5% (OBACK 2007; WHITWORTH & PRATHER 2010). Both cloned embryos as well as live-born cloned animals display a deregulation of the epigenetic state of their chromatin (WHITWORTH & PRATHER 2010). It is widely accepted that all of these defects are mainly related to the insufficient reprogramming of the chromatin of the donor nucleus in the cytoplasm of the metaphase II oocyte.

The procedure of somatic cloning used at present by most laboratories utilizes the fully differentiated metaphase II oocyte as the recipient for the donor cell nucleus. However, the study by BAO and colleagues (2000) suggests that full developmental competence of the oocyte chromatin is attained already in GV stage oocytes shortly before finishing their growth phase starting from oocytes at around 50-60 micrometers in diameter. Therefore, some key processes required to complete the reprogramming of the chromatin may occur before the oocytes reaches the metaphase II stage. The nature of these processes as well as how they are inducted, are unclear. It is possible that crucial activities such as those responsible for the completion of reprogramming in growing oocytes may already be depleted in metaphase II oocytes, and thus cannot participate in the reprogramming of the donor chromatin which in turn impairs the development of cloned embryos. For example, in the mouse the amount of mRNAs coding for Dnmt1 and Dnmt3L drastically decrease as the oocyte progresses from GV to metaphase II stage (VASSENA *et al.* 2005). Therefore, knowledge on chromatin modifications occurring in prophase oocytes during their growth and after its completion has not only a basic character but may also help to improve the procedure of somatic cloning.

We describe several changes in chromatin status of growing mouse oocytes, namely, progressive methylation of DNA and acetylation of histone H4, as well as a slight decrease in methylation of histone H4Arg3. The increase in global DNA methylation and histone H4 acetylation was described previously in growing oocytes of prepubertal mice (KAGEYAMA *et al*. 2007). Thus, our results indicate that in the adult mice these changes occur in a similar way. Our confirmation of these earlier results (KAGEYAMA *et al*. 2007) is very important since according to the general rules of the epigenetic code, a high level of DNA methylation and a high level of histone acetylation usually do not coexist (ALLIS *et al.* 2007). This suggests that the epigenetic phenomena related to the reprogramming of chromatin in the course of oogenesis may have a unique character.

According to a previous report, histone H4 Arg3 methylation is required for histone acetylation and maintenance of an active chromatin domain (HUANG *et al.* 2005). We have found a constant high level of this modification in growing oocytes with a slight decrease only at the transition between NSN to SN chromatin. This decrease may result from attaining a high level of histone H4 acetylation at the end of the growth phase since it was shown previously that histone H4 hyperacetylation inhibits methylation at arginine 3 (WANG *et al*. 2001).

In this study we have carefully selected oocytes according to their developmental stage by grouping them into categories differing in diameter by 5 μm. This enabled us to follow in detail the dynamics of chromatin modification with a resolution allowing to compare them with the timing of the acquisition of full developmental competence shown previously (BAO *et al.* 2000). In adult mice, growing oocytes with a diameter around 50-60 μm represent the earliest stage at which oocyte chromatin is able to support development upon transplantation into a fully grown enucleated oocyte (BAO *et al.* 2000). Our study reveals that this process may be associated with the alteration in global DNA methylation as we observed a significant increase in methylation between 55-59 and 60-64 μm oocyte groups. Subsequently global methylation increases gradually until the oocytes reach the final stage, consistent with results reported previously (BAO *et al*. 2000) in which a gradual increase in the ability of chromatin to support full development was observed. Our analysis shows that the increase in histone H4 acetylation follows the changes in DNA methylation occurring only slightly later, thus the involvement of this epigenetic modification in the acquisition of full developmental potential should also be considered.

The alterations in the status of histone H4 Arg3 methylation as well as the changes in localization in AKAP95 occur at the very end of the growth phase. Thus, these changes coincide with two processes – the silencing of oocyte chromatin (DE LA FUENTE & EPPIG 2001) and the reorganisation of chromatin conformation from NSN to SN type (DEBEY *et al*. 1993; ZUCCOTTI *et al*. 1995). It is known that transcriptional silencing and NSN-to-SN chromatin configuration change are independent (DE LA FUENTE *et al*. 2004). Whether any of these processes may be affected by histone H4Arg3 methylation or AKAP95 remains to be determined. Noteworthy, AKAP95 is involved in the organisation of chromatin (EIDE *et al.* 2002).

The low efficiency of somatic cloning results most probably from inappropriate/incomplete reprogramming of the donor chromatin. To examine the nature of this ineffectiveness, studies on chromatin modifications occurring immediately after nuclear transfer are necessary. We have addressed this issue by transplanting male pronuclei into fully grown enucleated GV stage oocytes followed by their culture. Since the global level of DNA methylation is high in the GV stage oocyte but low in the G2 male pronucleus, this experiment should show whether the recipient oocyte is able to change the status of DNA methylation of donor chromatin. Our results are negative. Moreover, the intact (not enucleated) fully grown GV stage oocyte were also unable to execute the DNA methylation of the hypomethylated DNA of growing oocytes. Thus, the inability to pursue DNA methylation does not seem to be specific to the type of donor nucleus transferred which may indicate a general failure of the recipient oocyte to carry out this process on foreign chromatin. This ineptitude may result from the removal of crucial proteins required for chromatin reprogramming along with the recipient oocyte nucleus, as shown for the GV-residing factors controlling the cell cycle (POLANSKI *et al*. 2005). However, we did not observe elevated DNA methylation in the growing oocyte nucleus upon its transfer into intact (not enucleated) fully grown GV oocytes. This could result, for example, from the sequestration of the involved activities in the GV of fully grown oocytes until germinal vesicle breakdown (GVBD), when the content of the prophase nucleus is released into the cytoplasm. Moreover, shortly after GVBD both the chromatin of the recipient oocyte as well as the foreign chromatin introduced by micromanipulation undergoes condensation (BALAKIER & CZOLOWSKA 1977; POLANSKI 1997) which may result in a dramatic reduction of its accessibility to the factors involved in DNA methylation. Additionally, post- GVBD depletion of some potential factors may also block the process, as shown for DNMT3L (VASSENA *et al.* 2005).

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