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Capturing Epigenetic Dynamics During Pre-implantation Development Using Live Cell Imaging

Kazuo Yamagata*

Laboratory for Genomic Reprogramming, Center for Developmental Biology, RIKEN-Kobe, Minatojima-minamimachi 2-2-3, Chuo-ku, Kobe city, 651-0047, Hyogo Japan

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During mammalian fertilization and pre-implantation development, the highly differentiated gametes revert to undifferentiated cell types following syngamy and then gradually differentiate into individual cell lineages. These processes involve changes in male and female gamete chromatin structure, in global epigenetic modifications and in nuclear architecture. We have developed a live cell imaging technique for oocytes and early embryos to understand these series of phenomena. Using this technique, we were able to observe dynamic changes in DNA methylation status in living embryos. Furthermore, epigenetic abnormalities were detected in reconstructed embryos generated by round spermatid injection or by somatic cell nuclear transfer. In this review, I will discuss the usefulness and possibilities of this imaging technique in studies on nuclear dynamics during fertilization and pre-implantation development.

Key words: DNA methylation, epigenetics, live cell imaging, pre-implantation development, somatic cell nuclear transfer.

NUCLEAR AND EPIGENETIC DYNAMICS DURING FERTILIZATION AND PRE-IMPLANTATION DEVELOPMENT

In multi-cellular organisms, all the cells in the body consist of only two cell types: somatic cells and germ cells. Although the sperm and the egg are highly differentiated cells in that they have specialized structures and functions, one can also argue that these cells are the most undifferentiated forms because all somatic cells derive ultimately from them. Therefore, germ cells have apparently contradictory properties being both 'terminally differentiated' and 'most undifferentiated', and such properties are never seen in all somatic cells. The transformation of germ cells from the differentiated to the undifferentiated state occurs suddenly. The process from fertilization to pre-implantation development might correspond to such a period. Indeed, the cells switch from being germ cells to totipotent zygote and then differentiate into various somatic cell lines. This process involves sequential multi-step events in the nucleus. Briefly, once a sperm enters the ooplasm, the oocyte, arrested at metaphase II, is activated. This triggers a number of morphological changes in the male and female nuclei, such as those involved in decondensation and re-condensation of the sperm nucleus, completion of second meiosis in the oocyte, formation of the male and female pronuclei and syngamy (Fig. 1). In addition to these structural changes in parental chromatin, it is known that drastic epigenetic alterations such as DNA demethylation (described below) and histone modification take place in the embryo after fertilization (*1–5*) (Fig. 1). Some recent reports have also described the dynamic regulation of the nuclear architecture (*6*) in the pre-implantation embryo, including chromatin configuration and organization (*7–9*). A set of such epigenetic and nucleic changes during this period is known to be associated with nuclear re-programming (Fig. 1), in which the sex-specific epigenetic 'memories' of the gametes are erased and those of the embryo are acquired gradually. It is conceivable that these alterations might affect global gene expression patterns and thus modify the properties of the cell lineages. Recent progress using somatic cell nuclear transfer (SCNT) to construct cloned embryos has revealed that the incomplete re-programming of the somatic cell genome memory in pre-implantation development can lead to aberrant phenotypes in the embryos and full-term animals (*10–12*). The efficiency of SCNT cloning is extremely low (*13*), and even if the embryos mature to adults, they often exhibit impaired phenotypes such as oversized placentae (*14*), immunological defects (*15*) and obesity (*16*). Clearly, correct re-programming during this period is required for normal embryonic development and ontogeny.

CHANGES IN DNA METHYLATION DURING PRE-IMPLANTATION DEVELOPMENT

DNA methylation involves the addition of a methyl group to the 5' position of the cytosine ring in CpG dinucleotides. It is catalyzed in mammalian cells by a family of highly related DNA methyltransferases that use *S*-adenosylmethionine as the methyl donor. Methylation of such regions might play a crucial role in the regulation of gene suppression such as X-chromosome inactivation, genomic imprinting and inactivation of transposable elements (*17–20*). Recently, increasing evidence has

*To whom correspondence should be addressed. Tel: +81-78-306-3094, Fax: +81-78-306-3095, E-mail: yamagata@cdb.riken.jp

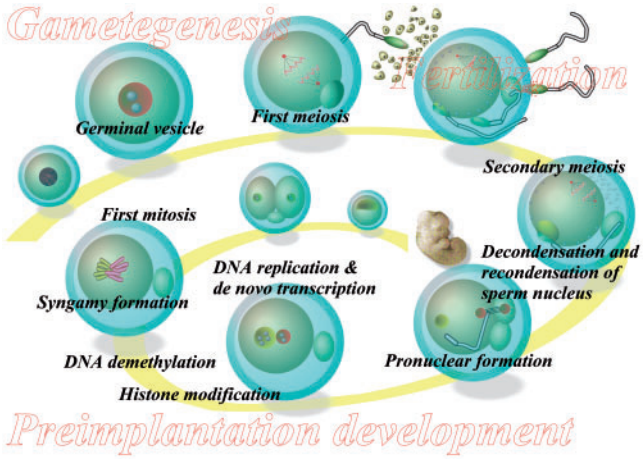

Fig. 1. **Nuclear and epigenetic dynamics during fertilization and pre-implantation embryonic development.** These phases of development entail major epigenetic alterations, including active and passive DNA demethylation and modifications to the nuclear histones. In addition, there are structural changes such as the decondensation and re-condensation of the sperm nucleus and the resumption of meiosis by the metaphase II chromosomes. The chromatin also undergoes configurational changes. *De novo* (zygotic) transcription starts at pronuclear stage.

revealed that DNA methylation of specific genomic regions during embryonic development might be closely related with cellular differentiation and lineage specification (*21–24*). As described, genome-wide changes in DNA methylation are thought to play important roles in the nuclear re-programming during pre-implantation development. Following fertilization, methylated zygotic genomes undergo both active and passive global DNA demethylation (*4*, *25*), and then the parental genomes acquire new methylation patterns specific for each cell type after embryonic implantation. On the other hand, embryos produced by SCNT occasionally exhibit aberrant patterns of DNA methylation in their pre-implantation stages (*26–29*). In addition, embryos cultured in suboptimal conditions fail to develop because of disordered methylation patterns (*30*). Thus, the precise regulation of DNA methylation in pre-implantation embryos might be essential for normal development. Therefore, we established a live cell imaging technique for early embryos to demonstrate the biological significance and mechanism of changes in DNA methylation in this period.

IMPORTANCE OF A LIVE CELL IMAGING TECHNIQUE IN STUDIES OF FERTILIZATION AND EARLY EMBRYONIC DEVELOPMENT

In mammals, the numbers of oocytes and embryos that can be collected are very limited. Therefore, the analysis of molecular mechanisms is hampered because of difficulties in conducting biochemical analyses on sufficient material. Also, immunostaining methods requiring cell fixation are insufficient for the understanding of ontogeny, because the processes observed in fertilization and early embryonic development progress in time-dependent manners, and each phenomenon is connected with others by cause-and-effect relationships. Consequently, it is important to develop an experimental system that enables one to obtain molecular imaging without affecting embryo development. Indeed, several studies have reported time-lapse observations of pre-implantation development to analyse the determinants of cell polarity (*31–33*). The advantages of live cell imaging are as follows.

(i) It allows observation of cells in a more natural state than conventional immunostaining techniques, which require troublesome procedures such as fixation and permeabilization.
(ii) Much more information can be made available by adding a temporal axis in seconds, minutes, hours or days.
(iii) It enables one to analyse the kinetics of molecules even in the early embryo by studying intermolecular interactions using Fluorescence Resonance Energy Transfer (FRET) and photomanipulation techniques such as Fluorescence Loss In Photobleaching (FLIP) and Fluorescence Recovery After Photobleaching (FRAP).
(iv) Molecular-level phenomena observed in early embryos can be connected directly with their developmental potencies by culturing and transferring the embryos to host mothers after fluorescent observations (retrospective and prospective analyses).

In particular, the retrospective and prospective analyses under item (iv) have been less described as an advantage of imaging techniques to date, but this is the most important and innovative aspect in the study of early embryonic development. For example, embryos constructed with particular reproductive technologies such as SCNT are considerably heterogeneous, and their developmental rates are not always constant (*34*). Furthermore, aged oocytes are reported be heterogeneous in terms of their developmental capacities (*35*). When such resulting embryos are immunostained, it is impossible to know the influence of any abnormality observed on subsequent development. However, live cell imaging allows us to link a specific phenomenon observed directly at a certain time to its developmental capacity by culturing and transplanting the embryo to a recipient pseudopregnant mother. Therefore, to exploit above advantages, especially retrospective and prospective analyses, we aimed to establish a live cell imaging system that would enable observations under minimally invasive conditions.

ESTABLISHMENT OF THE LIVE CELL IMAGING SYSTEM FOR PRE-IMPLANTATION EMBRYOS

To express any exogenous protein in cultured cells, it is common to use transfection with plasmid DNA followed by transcription and translation of the protein. For example, when using green fluorescent protein (GFP), it takes ~48–72 h to obtain sufficient fluorescence signals. Moreover, for mouse embryos, one has to wait

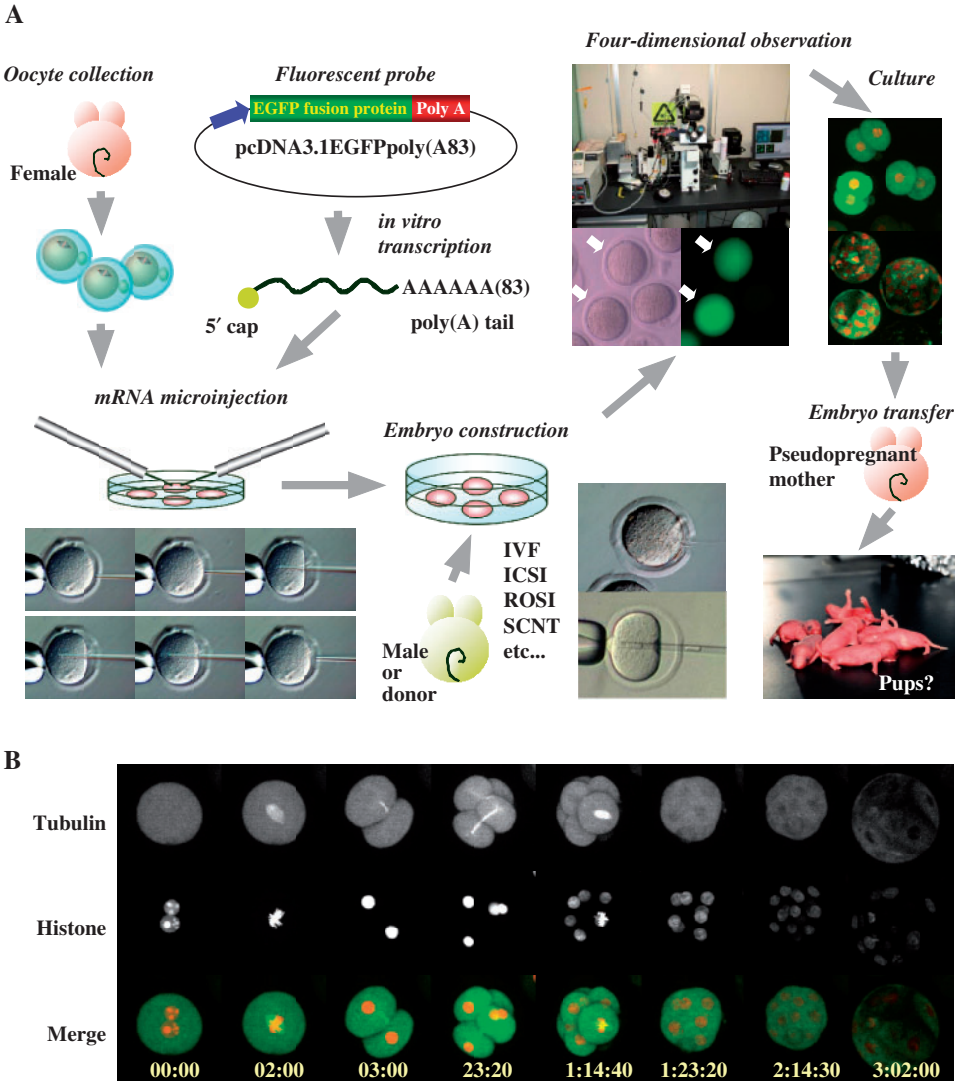

Fig. 2. **Live cell imaging of pre-implantation embryos.** (A) In our system, mRNA of fluorescent probe with an elongated poly(A) sequence and a 5' cap structure, is transcribed *in vitro* and then injected into the oocytes. Fluorescent signals can be obtained by 3 h after the mRNA injection. Embryos are constructed by various reproductive technologies. After four-dimensional (*x*, *y*, *z* and time) observations in the incubator on the microscope stage, the embryos are cultured further and, when necessary, transferred to pseudopregnant females to test their ability to develop for full-term offspring. (B) Time-lapse image of spindle and nuclei formations during pre-implantation development. The normally fertilized embryos were injected with a mixture of mRNAs encoding for fluorescent-labelled histone and for tubulin, and observed. Fluorescent images in each developmental stage are selected and represented.

until the 2-cell stage at the earliest to obtain measurable fluorescent signals, even if powerful promoters are used (*36*). However, it is not possible to monitor phenomena just after fertilization using this strategy. Alternatively, it is possible to generate transgenic animals that express GFP-fused proteins in the oocyte (*32*, *33*), but this requires expensive facilities to maintain the mouse strains. Therefore, we decided to use a technique to inject mRNA synthesized *via in vitro* transcription into the oocyte cytoplasm (*37*). Because translational efficiency depends on the length of poly(A) chain of mRNA in oocytes (*38*), our plasmid was designed to have a longer poly(A) sequence at the 3'-end of the mRNA transcribed *in vitro*. Furthermore, for *in vitro* transcription, the addition of a cap structure at the 5'-end enhances the translation of exogenous mRNA in the oocyte. In our imaging system (*39*), such *in vitro* synthesized mRNA was microinjected into unfertilized or fertilized oocytes, followed by insemination using *in vitro* fertilization or other reproductive techniques as necessary. After that, the constructed embryos were observed by fluorescent microscopy while being cultured inside a CO_2 incubator placed on the stage. Then, observed embryos were transplanted into pseudopregnant females, and their developmental capacities were determined (Fig. 2A). To detect fluorescence signals, a Nipkow-disk-type confocal unit was added onto the inverted microscope, and an ultrasensitive electron-multiplying

charge-coupled device (EM–CCD) camera was used for image capture. Using this system, we were able to capture four-dimensional fluorographic images (three dimensions over three days) to record cell division in pre-implantation embryos expressing fluorescence-labelled histone and tubulin (Fig. 2B). This showed that our imaging system was minimally invasive for studying early embryonic development.

IMAGING OF CHANGES IN GLOBAL DNA METHYLATION STATE DURING PRE-IMPLANTATION DEVELOPMENT

Most analyses of DNA methylation status in early embryonic development have been performed using immunostaining with antibodies to methylcytosine. This method requires the cell to be fixed and permeabilized. In addition, DNA fragmentation using strong acid treatment is required to enhance the affinity of the antibody. Hence, this method is suitable for the detection of global methylation levels in the nucleus, but it cannot provide the precise localization of methylated DNA because the chromatin structure itself is destroyed by this treatment. Therefore, we tried to capture changes in the global DNA methylation status of male and female chromatin in early embryos using live cell imaging (*7*, *23*, *39*). A construct (EGFP-MBD-NLS) was generated by fusing methylcytosine-binding domain and nuclear transport signal of human methylcytosine-binding protein (MBD1) with enhanced GFP (EGFP) (*40*). This was used as a fluorescent probe. Specific binding of this fusion protein to methylated DNA in embryos was confirmed by various *in vitro* and *in vivo* assays (*7*). First, time-lapse observation was performed, focusing on male pronucleus formation immediately after fertilization. Before insemination, the EGFP-MBD-NLS fusion protein was produced in metaphase II oocytes by injection of the corresponding mRNA. Unlike previous observations using an anti-methylcytosine antibody (*4*, *25*), no significant attenuation of the methylation level was detected in the pronucleus. Instead, intense EGFP-MBD-NLS fluorescence was observed in the central part of the decondensing sperm nucleus immediately after fusion between sperm and oocyte (60 min after insemination). This intense signal was retained in the enlarging male pronucleus until 3–4 h after insemination; it dispersed throughout the pronucleus at the 5 and 6 h stages, aggregated at the 7 h stage and then moved to the rim of the nucleolus. We next examined the changes in localization of methylated DNA in each stage of pre-implantation embryos. In the single-cell (late pronuclear) stage, there was a clear difference in the pattern localization of methylated DNA between male and female pronuclei. In the male pronucleus, most of the DNA methylation signals were observed around the nucleolus. By contrast, in the female pronucleus, fine dots of signals were seen dispersed in the entire nucleus as well as around the nucleolus (Fig. 3A). The numbers and sizes of the dot-like fluorescent signals of methylated DNA changed dramatically during the transition from 2-cell to 4-cell embryos. Fluorescence was detected as small dotted signals ($\sim$0.5 μm^2) in the nucleoplasm and as ring-like signals around the nucleoli of embryos at the 1-cell and 2-cell stages. However, the embryos at the 4-cell, 8-cell and morula stages only contained dotted EGFP signals that were bigger than those at the 1-cell and 2-cell stages (>2 μm^2), suggesting that the methylated DNA might aggregate to form clusters, likely corresponding to heterochromatin. Thus, during this very short period of embryogenesis, there are drastic changes in the configuration and architecture of DNA methylation in the nucleus, in addition to changes in the degree of methylation.

IMAGING OF DNA METHYLATION STATE IN EMBRYOS PRODUCED BY ROUND SPERMATID INJECTION (ROSI) AND BY SCNT

As described above, the developmental capacity of embryos reconstructed using reproductive technologies does not reach that of normally fertilized oocytes, for technical and biological reasons. To address these issues, we applied our imaging technique to such reconstructed embryos, and we performed retrospective and prospective analyses. We first applied it for the detection of DNA methylation status in embryos reconstructed using ROSI. In this approach, haploid round spermatids, progenitor cell of mature sperm during spermatogenesis, are microinjected into metaphase II oocytes—one of many possible types of assisted reproductive technology (ART) (*41–43*). In mature testicular sperm, the paternal genome is highly condensed with protamines, which are sperm-specific nuclear proteins. In contrast, the main non-DNA components of the immature round spermatid nucleus are histones, and the chromatin structure at this early stage of spermatogenesis is close to that of somatic cells. Importantly, aberrant DNA methylation status has been reported in ROSI-produced embryos using immunostaining for methylcytosine (*44*). Therefore, we aimed to test for associations between any aberrancy in DNA methylation and the developmental potential for each embryo. ROSI was performed on metaphase II oocytes, and EGFP-MBD-NLS mRNA was injected. Fluorescent signals were then detected in each pronuclear stage embryo. Intra cytoplasmic sperm injection (ICSI)—injection of the isolated mature sperm head into the ooplasm—was used as a control mode of fertilization. Although the patterns were similar, a small but significant increase in DNA methylation around the nucleolus was detected in ROSI embryos compared with ICSI embryos (Fig. 3B). In addition, abnormal localization of methylated DNA in the nucleus was observed frequently in ROSI embryos. Most of the signals were found around the nucleolus of the male pronucleus in ICSI embryos, whereas some ROSI oocytes contained small, bright dots scattered in the male nucleoplasm (Fig. 3C and D, yellow arrows). We then determined whether these abnormalities in methylated DNA of ROSI embryos could affect their pre-implantation development by retrospective analysis. After observation of fluorescence at the pronuclear stage, each embryo was cultured individually in droplet culture for 72 h, and its capacity for blastocyst formation was analysed. This showed that developmental potential was affected by abnormalities in the localization of methylated DNA within the male pronucleus, rather than by the absolute intensity of methylation around

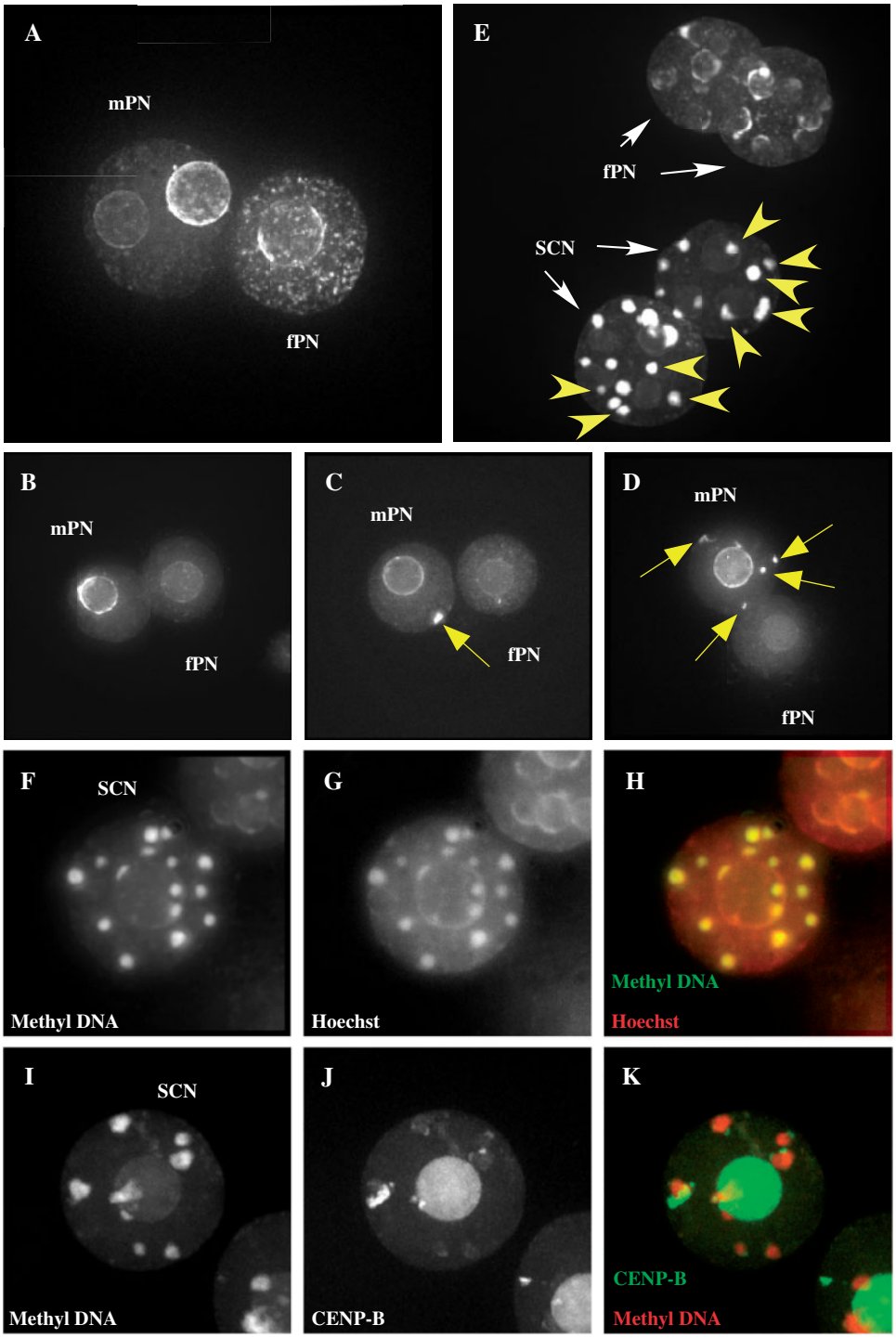

Fig. 3. **DNA methylation pattern in pronuclear-stage embryos.** Oocytes were first microinjected with EGFP-MBD-NLS mRNA, and then embryos constructed by *in vitro* fertilization (A), by ROSI (B–D) or by SCNT of cumulus nucleus (E–K) were observed using fluorescence microscopy at the late pronuclear stage. mPN, fPN and SCN indicate the male pronucleus, female pronucleus and somatic cell nucleus, respectively. Panels F–K show somatic cell nuclei in SCNT embryos co-stained with Hoechst 33342 (F–H) or CENP-B (I–K).

the nucleolus. Thus, the proper configuration of methylated DNA in the nucleus of the early embryo is required for normal development. Moreover, the chromatin structure of the male genome is controlled precisely in embryos and in spermatogenesis. It is very interesting that the state of DNA methylation in ROSI embryos differed from the control ICSI embryos, given that the molecular mechanisms of global demethylation of DNA

normally occur after fertilization (*4*, *25*). Thus, epigenetic error seen in ROSI embryos may be associated with the aberrant gene expression pattern in those embryos (*45*) and with their poor developmental capacities (*41*, *46*). Moreover, this also provides an important insight into the potential risks of ROSI as an ART to resolve severe human male infertility (*47*).

Finally, DNA methylation status in SCNT (cloned) embryos was analysed by our imaging technique (*23*). In SCNT, only a few percent of cloned embryos can develop normally (*13*). However, treatment of SCNT embryos at the pronuclear stage with the histone deacetylase inhibitor trichostatin A (TSA) can improve the efficiency of cloning (*48*). This suggests that some epigenetic modification following DNA methylation, including histone modification, promotes the genomic re-programming process in SCNT procedure. Indeed, several reports have described abnormalities in DNA methylation status in SCNT embryos using immunostaining (*26–28*), and by bisulphite sequencing of particular repetitive elements (*29*) and gene promoter regions (*49*). In other words, it is suspected that most of such embryos do not undergo complete re-programming and that this causes developmental failure. Therefore, to understand the mechanism of re-programming in cloned embryos, it is important to observe the state of DNA methylation in the somatic nuclei used for construction and to relate this directly to individual developmental capacity. Cumulus cell nuclei were transferred to metaphase II oocytes previously microinjected with EGFP-MBD-NLS mRNA. Although routine SCNT cloning involves removal of the metaphase II chromosome plate, in this case, this was not done to allow a direct comparison of the state of DNA methylation between the somatic cell nucleus and the recipient oocyte nucleus. The somatic cell nucleus had a significantly higher level of methylation than that of the oocyte (Fig. 3E, arrowheads). The highly methylated region in the somatic nucleus possibly corresponded with the centric/pericentric region of the chromosomes because the methylated signals co-localized with Hoechst 33342 staining (Fig. 3F–H) and were located adjacent to CENP-B (Fig. 3I–K), one of the centromeric proteins. Time-lapse observations showed that this high methylation level in the somatic cell nucleus was maintained up to the first cell division. Another line of our experiment revealed that the highly methylated status in centric/pericentric region of cloned embryos persisted throughout pre-implantation development (*23*). Based on these results, we conclude that the highly methylated regions in somatic cell nuclei, most likely the centromeric region, do not undergo re-programming by the active and passive demethylation mechanisms known to be present in pre-implantation embryos (*4*, *25*). In other words, this incomplete re-programming of DNA methylation in the centromere may cause the developmental abnormalities observed in cloned embryos. However, as described above, a few cloned embryos can undergo full genomic re-programming and develop to full term. Using our live cell imaging technique, we would like to be able to determine which cloned embryo has the potential to develop, by selecting embryos and observing the result in each case. This approach is likely to shed light on the low success rate of current SCNT cloning technology.

CONCLUSIONS AND PERSPECTIVES

We have established a new experimental system that enables us to observe the molecular events in living oocytes and early embryos: it has many advantages over conventional immunostaining. Here we have focused on our results using time-lapse and retrospective analysis of the state of DNA methylation during the pre-implantation development of normal embryos and those produced by ICSI, ROSI and SCNT cloning. We have also analysed the kinetics of HP1 beta protein changes in the euchromatin and heterochromatin of pre-implantation embryos using FRAP assay (*50*). Through these observations, we have succeeded in capturing dynamic changes in the epigenetic status and chromatin configurations in the living embryos for the first time, as these observations cannot be recognized by conventional immunostaining. Furthermore, the combination of this approach with loss- or gain-of-function experiments, such as co-injection with exogenous proteins, with dominant negative forms of mRNA or with interfering RNA, would allow us to manipulate the embryo to gain further insights at a molecular level. From an ethical standpoint, it is worth emphasizing that the offspring generated from embryos injected with mRNA for imaging purposes are not 'transgenic', because this method does not integrate exogenous genes into the chromosomes. Therefore, this technique is also potentially applicable to animal industry and even to human ART as a method for selecting 'good-quality' embryos before implantation.

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