

—Mini Review—

**“Nuclear Reprogramming” and
“Epigenetic Reprogramming”**Takashi Tada^{1*}, Hironobu Kimura¹ and Masako Tada^{1,2}¹Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan²ReproCELL inc., 1-1-1 Uchisaiwai-cho, Chiyoda-ku, Tokyo 100-0011, Japan**Introduction**

“Nuclear reprogramming” is a phenomenon regulated by complex mechanisms that lead to the restoration of pluripotential competence in specialized somatic nuclei. Nuclear reprogramming is induced by changes in epigenetic modifications, known collectively as “epigenetic reprogramming”. In somatic cell development, on-off switching of certain key genes, which function in determining cell fate in a particular direction, is regulated through epigenetic reprogramming in restricted regions of the genome. In nuclear reprogramming, genome-wide epigenetic reprogramming, which triggers a global restoration of epigenetic memory in the genome leading to transformation from a specified to a default nuclear state, is crucial. Genome-wide epigenetic reprogramming occurs in nuclear reprogramming with the nuclear transfer of somatic cells to enucleated oocytes and via cell hybridization between embryonic stem cells and adult somatic cells, and also in germ cell and early embryonic development but not in somatic cell development. Global chromatin de-condensation marked by histone H3 lysine 4 methylation is mechanistically linked with the genome-wide epigenetic reprogramming. At least two steps; 1) erasure of the somatic epigenotype induced by the genome-wide epigenetic reprogramming and 2) establishment of a pluripotential cell-specific epigenotype by local epigenetic reprogramming through the activity of key players including *Oct4*, *Sox2*, *Ehz2* and *Nanog*, may be required for conferring and maintaining pluripotential competence in the reprogrammed somatic nuclei.

**Nuclear Reprogramming in
Early Embryonic Development**

Our body is built of an incredible variety of cell and tissue types, which develop from a single fertilized egg through embryogenesis. Cells are basically classified into two types: somatic cells and germ cells. Somatic cells function in forming and maintaining body parts only for one generation, whereas germ cells including gametes and their precursor cells are diversified for transmitting genetic information to the next generation. Remarkably, in germ cell and early embryonic development but not in somatic cell development, two waves of genome-wide epigenetic reprogramming take place in primordial germ cells (PGCs) when entering the gonads and in the preimplantation embryos, respectively [1]. The PGC-specific genome-wide epigenetic reprogramming plays an important role in resetting parental epigenetic memories (including imprints), whereas the early embryo-specific genome-wide epigenetic reprogramming functions to erase the gamete-specific epigenetic memories (excluding imprints) required for equalizing the function of the paternal and maternal genome as detected by global DNA de-methylation and histone acetylation [2, 3].

A germ cell determinant, germ plasm (polar plasm) is accumulated as a maternal factor in the eggs of *Drosophila melanogaster*, *Caenorhabditis elegans* and *Xenopus laevis*, but it is believed that germ plasm does not exist in mammalian eggs [4], suggesting that germ cell and somatic cell lineages are not determined in the early embryos. In the mouse, PGCs are first identified as alkaline phosphate-positive cells clustering in the proximal region of allantois of the E7.25 embryo [5]. The emergence of these PGCs is induced by the synergistic action of extrinsic signals, BMP4 and BMP8B, secreted from the extraembryonic ectoderm

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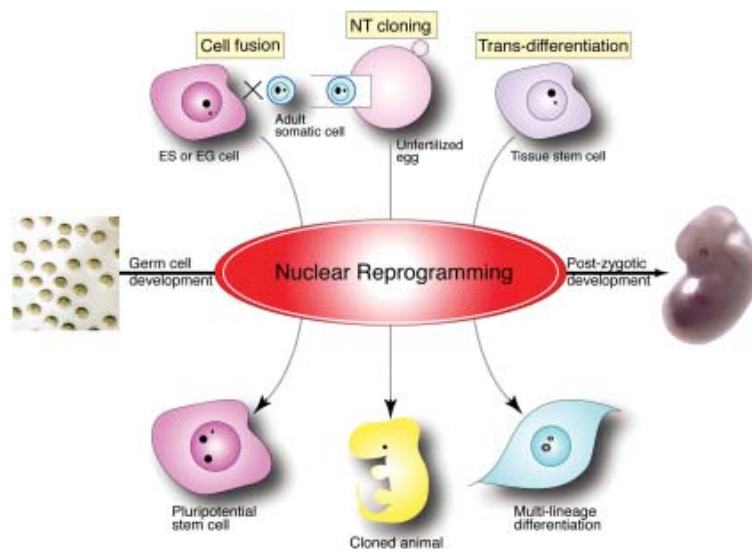


Fig. 1. Nuclear reprogramming *in vivo* and *in vitro*. NT: nuclear transplantation. ES: embryonic stem. EG: embryonic germ.

adjacent to the epiblast of E5.5 embryos [6, 7]. Furthermore, *Smad5* is involved in PGC generation and localization as a signal mediator downstream of the BMP pathway [8]. The hypothesis is proposed that germ line-competent cells are marked by an interferon-inducible transmembrane protein, *Ifitm3* (*Fragilis, mil-1*), in the E6.0-E7.5 embryos [9, 10] and then PGCs are specified by reduction in *Ifitm3* and subsequent increase in *Stella/PGC7* [9, 11] and *Tnap* (Tissue non-specific alkaline phosphatase) [12] in the E7.25-7.5 embryos. In the PGCs specified by the marker genes, the parentally inherited epigenotype is retained. Based on the kinetics of DNA methylation of imprinted and non-imprinted genes, the genome-wide epigenetic reprogramming is initiated in the migrating PGCs of E9.5 embryos and subsequently completed soon after their entry into the genital ridges in both E12.5 male and female embryos to build an equivalent epigenetic state prior to the diversification to male and female gametes [13–20]. Reactivation of the inactivated X chromosome in female PGCs also occurs in the gonads of E11.5-12.5 embryos [21, 22]. After fertilization, sperm-derived chromatin is drastically transformed by the activity of the nucleoplasmin due to the exchange of sperm-specific basic proteins (protamines) to maternal acetylated histones [23], resulting in the onset of the equalization of paternal and maternal genomes. Post-zygotic genome-wide DNA de-methylation takes place in early cleavage stages and DNA re-methylation is linked to the

first-lineage cell differentiation during mammalian development [2]. Thus, genome-wide reprogramming is intrinsically linked to nuclear reprogramming in the germ cell and preimplantation development (Fig. 1), but the molecular mechanisms of nuclear reprogramming are largely unknown.

Nuclear Reprogramming via Nuclear Transplantation of Somatic Cells

The nuclear reprogramming activity of oocytes was first demonstrated with the successful production of cloned frogs by nuclear transplantation of the intestinal endoderm cells of feeding tadpoles into activated enucleated eggs [24]. More recently, nuclear transplantation of specialized somatic cells into enucleated oocytes demonstrated that mammalian oocytes also retain a nuclear reprogramming capacity as shown by the production of cloned animals such as sheep, cows, mice, pigs, cats, rats and monkeys [25, 26] (Fig. 1). Thus, amphibian and mammalian oocytes share an intrinsic nuclear reprogramming activity, and at least in part, reprogramming molecules and mechanisms, but the primary loss of eggs receiving transplanted nuclei of somatic cells differs in Amphibia and mammals. In mammals, the developmental loss occurs during relatively late stages, whereas in Amphibia, cloned eggs stop dividing in the early cleavage stages, suggesting that these temporal

variations in embryonic loss probably reflect different difficulties encountered after the nuclear transplantation of somatic nuclei in Amphibia and mammals [27]. In mammals, it has been suggested that the developmental loss of cloned embryos could be caused by insufficient nuclear reprogramming of somatic nuclei. This is supported by the observed expression of somatic nucleus-derived *Oct4-GFP* in a limited number of cloned blastocysts [28], aberrant reactivation of *Oct4*-related genes [29], and abnormal hyper-methylation of histone H3 lysine 9 (K9) associated with DNA hyper-methylation in cloned preimplantation embryos [30, 31]. Another interesting suggestion is that developmental failure is due to a technical factor associated with enucleation with the maternal spindle proteins required for maintaining ploidy through the initial cleavages [32]. But it is largely unclear what the mechanisms and key molecules responsible for insufficient nuclear reprogramming and full nuclear reprogramming are.

Nuclear reprogramming of adult somatic cells or tissue stem cells is of interest as a procedure for generating personalized pluripotent stem cells, which is the ultimate cell source (stem cells of the second generation) of therapeutic materials for use in regenerative medicine. The personalized stem cells are pluripotent, self-renewing and immunologically syngeneic to the original somatic cell (the somatic cell donor). Therefore, the immune system of the somatic cell donor will be completely tolerant of derivatives from personalized stem cells. In fact, mouse embryonic stem (ES) cells derived from cloned blastocysts created by nuclear transplantation of somatic cell nuclei have been produced [33–35]. Furthermore, as a therapeutic model system in the mouse, the phenotype of immunodeficient *Rag2(-/-)* is rescued by grafting hematopoietic precursors generated from cloned ES cells with gene therapy with the wild-type *Rag2* gene [36]. Recent developments in the field of stem cell research include the successful generation of mouse sperm and oocytes from ES cells in culture [37–39]. A combination of these newly established technologies may facilitate cell replacement with less ethical issues.

Nuclear Reprogramming via Cell Fusion with Embryonic Stem Cells

Mouse ES cells derived from the inner cell mass cells of blastocysts are defined by almost indefinite proliferation under appropriate culture conditions, without a loss of differentiation potential, into the cell types of adult somatic and germ cells [40, 41]. The

successful establishment of human ES cells from blastocysts has also been reported [35, 42, 43]. An important finding is that ES cells have an intrinsic capacity for the epigenetic reprogramming of somatic genomes after cell fusion [44–46] (Fig. 2). In hybrid cells between ES cells and adult thymocytes, nuclear reprogramming of somatic genomes has been shown by 1) the contribution of ES hybrid cells into normal embryogenesis of chimeras, 2) the reactivation of the silenced X chromosome derived from a female somatic cell, 3) the reactivation of pluripotent cell-specific genes, *Oct4*, *Xist* and *Tsix*, which are derived from a somatic cell, 4) the re-differentiation down variety of cell types in teratomas, 5) the tissue-specific gene expression from reprogrammed somatic genomes in addition to ES genomes *in vivo* in differentiated teratomas and *in vitro* in differentiated neuronal cells and 6) the acquisition of pluripotent cell-specific histone-tail modifications in reprogrammed somatic genomes. More interestingly, cell fusion experiments on somatic cells and embryonic germ (EG) cells, particularly those derived from gonadal primordial germ cells of mouse 11.5–12.5 dpc embryos demonstrate that EG cells possess additional potential for inducing reprogramming of somatic cell-derived parental imprints accompanied by a disruption of the parental-origin-specific DNA methylation of imprinted genes [16, 47]. Therefore, cell fusion with pluripotent stem cells is now recognized as an effective approach to elucidating the mechanism of nuclear reprogramming concerned with DNA and chromatin modifications.

More recent evidence shows that neurosphere and bone marrow cells will undergo nuclear reprogramming after spontaneous cell fusion, when co-cultured with ES cells *in vitro* [48, 49]. *In vivo*, the contribution of spontaneous cell fusion to the regeneration of tissues has been proven by the transdifferentiation of bone-marrow-derived cells as hybrid cells into Purkinje neurons, cardiomyocytes and hepatocytes [50–53]. Thus, the nuclear reprogramming of somatic cells by *in vivo* cell fusion plays an important role in maintaining the homeostasis of some tissues through regeneration during defined self-renewal and after tissue damage, although not in all cases of spontaneous tissue repair [54, 55].

Molecular Mechanism of Nuclear Reprogramming

Nuclear reprogramming is regulated by complex mechanisms that lead to the restoration of pluripotent

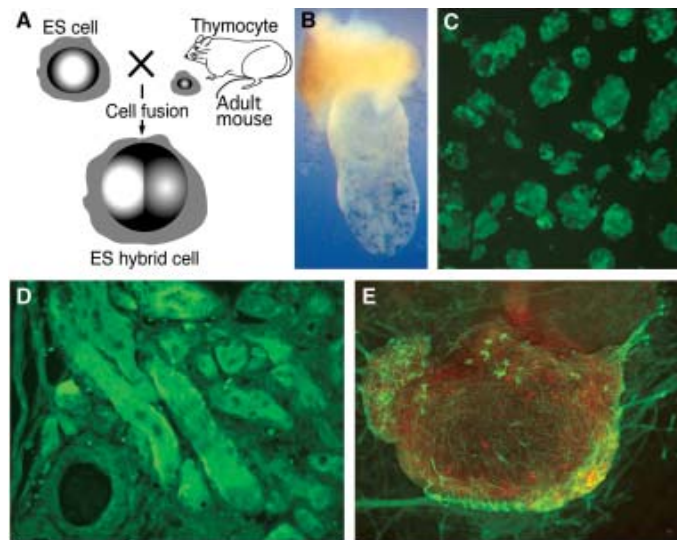


Fig. 2. Nuclear reprogramming of somatic nuclei by cell fusion with embryonic stem (ES) cells. (A) Cell fusion between ES cells and thymocytes derived from adult mice. (B) A representative E7.5 chimeric embryo with ES hybrid cells. ES hybrid cell derivatives are visualized as blue cells by X-gal staining. (C) Reactivation of the *Oct4-GFP* reporter gene in ES hybrid cells. The *Oct4-GFP* reduced in somatic cells is increased and stably expressed after cell fusion with ES cells. (D) *In vivo* differentiation of ES hybrid cells in teratoma. ES hybrid cells differentiate to muscle cells positive for immunoreaction with anti-Desmine antibody. (E) *In vitro* differentiation of ES hybrid cells. Neurofilament-positive (green) and tyrosine hydroxylase-positive (red) cells are efficiently generated from ES hybrid cells by co-culture with PA6 stromal cells.

competence in specialized somatic nuclei. Spontaneous transformation from somatic cells to pluripotential stem cells has not been found in vertebrates, suggesting that the process of nuclear reprogramming may be firmly prohibited and consist of complicated multi-step events. In Amphibia, somatic nuclei injected into an oocyte underwent a large increase in volume and dispersion of their chromatin [27]. Furthermore, new protein synthesis from the injected mouse and human nuclei is induced in *Xenopus* oocytes [56]. In mouse germ cell development, an enlargement of the nucleus in volume is classically observed in PGCs soon after entry into genital ridges at the stage when the PGC-specific genome-wide epigenetic reprogramming takes place. These findings indicate that the chromatin of somatic nuclei may be de-condensed after nuclear reprogramming.

During nuclear reprogramming, it is expected that DNA and chromatin modifications should be

significantly effected. As epigenetic modifications, replication timing, DNA cytosine methylation, histone acetylation, histone methylation, histone variant, phosphorylation, ubiquitination, sumoylation, glycosylation and ADP ribosylation are believed to be involved in regulating the transcriptional activation or repression of genes. The histone modifications are thought to play certain key roles in regulating gene activity, most likely through the modification of the chromatin structure [57]. Regarding histone acetylation and methylation, to date at least eight acetyltable lysine positions are known in the N-termini of histones H3 (K9, K14, K18, K23) and H4 (K5, K8, K12, K16) and six methyltable lysine positions exist in those of histones H3 (K4, K9, K27, K36, K79) and H4 (K20). In general, the acetylation of histones H3 and H4 correlates with gene activation, while deacetylation correlates with gene silencing [58]. The methylation of H3-K4 also marks active chromatin, which contrasts with the modification of inactive chromatin by the

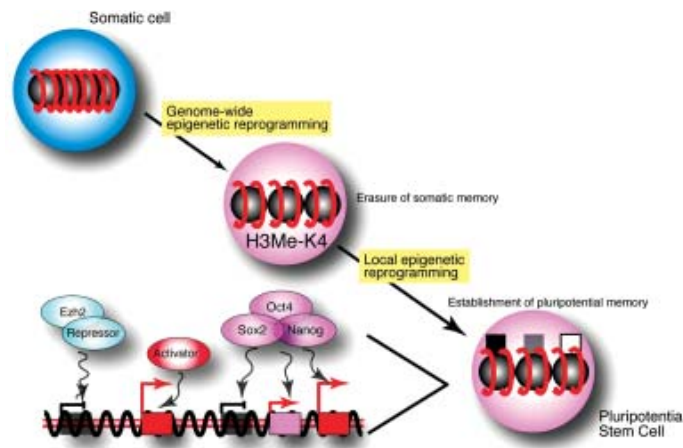


Fig. 3. Nuclear reprogramming of somatic nuclei through two distinguishable events. The first event is the erasure of somatic memory due to genome-wide epigenetic reprogramming. The second event is the establishment of pluripotential cell-specific memory built by local epigenetic reprogramming. H3Me-K4; histone H3 methylation at lysine 4.

methylation of H3-K9 [59]. The methylation of H3-K27 is an epigenetic marker for the recruitment of the polycomb group (Pc-G) complexes [60] and is prominent in the inactivated X chromosome of female mammalian somatic cells [61, 62]. The amino-terminal tail of histone H3 is subject to three distinctive methylation states, mono-, di- and tri-methylation. Pericentric heterochromatin is enriched in tri-methylated H3-K9, whereas centromeric regions are enriched in di-methylated forms [59]. At H3-K27, both di- and tri-methylation are observed across several nucleosomes, and it is the tri-methylated form that has been found to induce the stable recruitment of Pc-G complexes [63]. At H3-K4, fully activated promoters are enriched with the tri-methylated form, whereas H3-K4 di-methylation correlates with the basal transcription-permissive state [64]. Therefore, it appears that di-methylation activity prepares histones for a tri-methylating activity, which then propagates stably activated or silenced chromatin domains.

In the somatic cell-derived nuclei fully reprogrammed in ES hybrid cells, post-translational histone acetylation and methylation of histone N termini are analyzed with a technique of chromatin immunoprecipitation. The reprogrammed somatic genome becomes globally hyper-acetylated at histones H3 and H4. Of note, histone H3-K4 become hyper-di and tri-methylated irrespective of the gene activity [65]. De-condensed chromatin marked by histone H3-K4 hyper-methylation

is globally formed by genome-wide epigenetic reprogramming. This event may be linked with the swelling of the nucleus in PGCs when genome-wide epigenetic reprogramming takes place. Global epigenetic reprogramming may lead to the formation of basal transcription activation-permissive chromatin and function in erasing pre-established somatic cell-specific epigenetic memory prior to the building of the pluripotential cell-specific epigenotype. Subsequently, the pluripotential stem cell-specific epigenotype is established by the repression of somatic cell-specific genes and activation of pluripotential cell-specific genes through local epigenetic reprogramming by the activities of pluripotential cell-specific key regulators such as *Oct4*, *Sox2*, *Ezh2* and *Nanog* [66–71]. Therefore, we propose that the nuclear reprogramming of somatic nuclei involves at least two distinct events, 1) erasure of somatic cell-specific epigenetic memory by genome-wide epigenetic reprogramming and 2) establishment and maintenance of a pluripotential cell-specific epigenotype by local epigenetic reprogramming (Fig. 3). In the second event, it is likely that the key genes work cooperatively through molecular communication in the stem cell-specific regulatory network.

Conclusion

Genome-wide epigenetic reprogramming is a crucial molecular event needed to induce sufficient nuclear

reprogramming in germ cell and early embryonic development *in vivo*, and also by nuclear transplantation of specialized somatic nuclei into unfertilized oocytes and cell fusion between ES or EG cells and adult somatic cells *in vitro*. Nuclear reprogramming technology is required for producing second generation stem cells, which are immunologically syngeneic pluripotential stem cells generated from personal somatic cells. Identification of the molecular mechanisms and factors implicated in the nuclear reprogramming will provide a clue to the mechanisms of succession of life from generation to generation. A greater understanding of these mechanisms will bring biomedical applications and basic science in the field of stem cell biology closer together.

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