Toti-/pluripotential Stem Cells and Epigenetic Modifications

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ABSTRACT. The recent fascinating breakthrough in the area of stem cell research is the successful production of cloned animals via nuclear transplantation of somatic nucleus by intrinsic *trans*-acting factors of oocytes and trans-differentiation of somatic stem cells from adult organs induced by extrinsic growth factors. During the process of nuclear reprogramming, epigenetic modification of the somatic nuclei must be achieved to acquire toti-/pluripotential competence. However, the molecular mechanism involved is largely unknown. It has been shown that DNA methylation, histone acetylation and chromatin structure are involved in the establishment of epigenetic modification. Now it is evident that they function cooperatively to establish and maintain active or inactive chromatin state. Here we discuss the mechanisms of epigenetic modification potentially involved in the event of nuclear reprogramming.

Key words: Stem cell/ES cell/EG cell/transdifferentiation/animal clone/nuclear transplantation/cell hybridization/DNA methylation/histone acetylation/chromatin structure/reprogramming

Recent developments in the field of stem cell research have been boosted by advances in cloning from somatic cells in mammals, the establishment of primate ES cells, and their differentiation to specific cell types such as dopamine-producing neurons and vascular progenitors for clinical applications in reproductive medicine (Kawasaki *et al.*, 2000; Yamashita *et al.*, 2000). Another rapidly growing field is the understanding of transcriptional regulation and epigenetic modifications, which include DNA methylation, histone acetylation and chromatin remodeling. The isolation and characterization of key molecules involved in these epigenetic modifications indicate that a variety of factors cooperate in the form of large complexes that direct the building of specific nucleosome conformations, which in turn regulate transcriptional activation and repression. Until

recently the fields of stem cell research and epigenetic research have been developed independently. However, it is now evident that these fields should no longer be viewed separately. It is likely that the mechanisms by which toti-/pluripotential stem cells maintain their characteristic nuclear state, which gives them the competence to differentiate into multiple cell types, is different to the regulatory mechanisms found in somatic cells. The epigenetic state of toti-/pluripotential stem cells and cloned somatic nuclei could be explained, at least in part, by the latest findings relating to chromatin remodeling. Here, we will present an overview of the research into stem cells and epigenetic modifications in an attempt to bring these two rapidly evolving fields closer together.

Pluripotential stem cells; ES and EG cells

Pluripotential stem cells of the mouse are continuously maintained in an undifferentiated state, and are capable of largely increasing in number through rapid cell divisions. Under appropriate conditions these cells will differentiate into ectodermal, mesodermal and endodermal derivatives in the formation of embryoid bodies following *in vitro* suspension culture, and in teratoma formation by *in vivo* transplantation (Martin, 1980). Classically, pluripotential stem cells

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Abbreviations: ES, embryonic stem; EG, embryonic germ; EC, embryonal carcinoma; PGC, primordial germ cell; HSC, hematopoietic stem cell; GFP, green fluorescent protein; Dnmt, DNA methyltransferase; MeCP, methylated-DNA-binding protein; MBD, methyl-CpG-binding domain; HAT, histone acetylase; HDAC, histone deacetylase; SWI/SNF, ATP-dependent chromatin remodeling complex.

were isolated from mouse teratocarcinomas, and were termed embryonal carcinoma (EC) cells (Pierce, 1967). Other cell lines, which have similar properties to EC cells were first isolated from the inner cell mass (ICM) of mouse blastocysts. Importantly, the production and isolation of these cells, originally termed EK cells (Evans and Kaufman, 1981), and later embryonic stem (ES) cells (Martin, 1981), did not require carcinogenesis (Fig. 1). ES cells maintain a normal karyotype through numerous cell divisions in culture, and will efficiently contribute to the somatic and germ cell lineages of chimeras following their reintroduction into recipient mouse blastocyst ICMs. In addition, homologous recombination will occur in mouse ES cells with very high efficiency. Thus, research using ES cells has provided enormous biomedical benefits through the *in vitro* manipulation of the genome, by allowing for a greater understanding of complex biological systems *in vivo* using transgenic and knockout mice (Capecchi, 1989). More recently a third type of pluripotent stem cell line, embryonic germ (EG) cells, was established from primordial germ cells (PGCs) in the mouse embryo, both migrating and shortly after entry into the gonads (Matsui *et al.*, 1992; Resnick *et al.*, 1992) (Fig. 1). Similar to ES cells, EG cells are able to efficiently colonize chimeric embryos, and their genomes can be successfully transmitted to the next generation (Labosky *et al.*, 1994; Tada *et al.*, 1998). However, the epigenetic state of

EG cells derived from gonadal PGCs is different to that of ES cells, and EG cell chimeras show phenotypic abnormalities, which include fetal overgrowth and skeletal malformation. This can be explained, at least in part, by the associated bi-allelic expression/repression of some imprinted genes, which are transcribed according to their parental-origin (Tada *et al.*, 1998). The three types of pluripotent stem cells, EC, ES and EG, resemble each other in phenotype, characterised by a large nucleus and small amount of cytoplasm, and the retention of a robust capacity for proliferation.

In other mammalian species, including rat (Vassilieva *et al.*, 2000), rabbit (Schoonjans *et al.*, 1996), pig (Shim *et al.*, 1997; Chen *et al.*, 1999), sheep (Notarianni *et al.*, 1991) and cow (Iwasaki *et al.*, 2000), ES-like and EG-like cells have been established for the purpose of cell-mediated transgenesis, to include the production of animal models for human diseases, bioreactors for production of human therapeutic proteins, organ donors for transplantation into humans, and livestock which produce valuable products with high efficiency. In the world of primates, ES cells have been successfully derived from blastocysts of the rhesus monkey (Thomson *et al.*, 1995), the common marmoset monkey (Thomson *et al.*, 1996) and the cynomolgus monkey (Suemori *et al.*, in press). The successful establishment of human ES cells from blastocysts (Amit *et al.*, 2000; Thom-

Fig. 1. Derivation of pluripotent stem cells. Nuclei of adult somatic cells acquire pluripotent competence by epigenetic reprogramming via cell hybridization with pluri-potent stem cells and nuclear transplantation to enucleated oocytes. Tissue-specific multipotent stem cells are capable of trans-forming to pluripotent stem cell by extrinsic stimulation as similarly seen by ES and EG cell derivation from the inner cell mass cells of blastocysts and primordial germ cells. Pluripotency of these cells is shown by differentiation to various types of tissues in chimeras.

son *et al.*, 1998), and human EG cells from PGCs of 5–9 week old embryos (Shamblott *et al.*, 1998) have also been reported. In conclusion, the ICM cells of mammalian blastocysts appear to have a common capacity for transformation into pluripotent stem cells when kept under appropriate culture conditions *in vitro*.

Transdifferentiation of somatic stem cells

The most powerful stem cell is the totipotent fertilized oocyte, which grows and differentiates into every type of complex, functionally distinct, multicellular tissue during whole body formation. Even after complete maturation some somatic cells retain stem cell-like properties. These are, however, restricted in their differentiating potential and play an important role in maintaining homeostasis of many tissues and organs by regeneration during defined self-renewal and following tissue damage. Primitive animals have a very powerful ability for self-regeneration. For instance, planarians and hydrozoans can rebuild a perfect body from small fragments, and some amphibians and reptiles can regenerate whole body parts, including the tail and limbs. In birds, some neurons retain the potential for regeneration. However, self-regenerative activity has, until recently, been less notable in mammals. Hair, skin, small intestine and hematopoietic cells chronologically divide, terminally differentiate, and die. It has therefore been long suspected that a subpopulation of somatic stem cells may be maintained in these tissues in mammals. Surprisingly, however, recent stem cell research has revealed that the ventricular system of the fore- and midbrain, hippocampus and the spinal cord also retain multipotent stem cells, as shown by the generation of neurons, glia and oligodendrocyte progenitor cells in culture (Temple and Alvarez-Buylla, 1999; Momma *et al.*, 2000). This contradicts the long held notion that mammalian neurogenesis ceases in embryonic and early postnatal development. The precise nature of adult neural stem cells, including their location, function and how their proliferation is regulated is, however, largely unknown. More surprisingly, adult neural stem cells retain the potential to differentiate into all germ layers, including various types of somatic tissue, in addition to the neural cell lineage (Clarke *et al.*, 2000). The neural stem cells were isolated *in vitro* as neurospheres from adult brains of ROSA26 transgenic mice, which carry the ubiquitously expressed *neo/lacZ* reporter gene. These were introduced into the amniotic cavity of early chick embryos and the blastocoel cavity of mouse blastocysts. X-gal staining of the chick and mouse manipulated embryos clearly showed that the adult neural stem cell derivatives contributed to many embryonic tissues, similar to that seen in ES cell chimeras. The neural stem cells therefore possess the potential to transdifferentiate into pluripotent stem cells.

This intriguing transdifferentiation phenomenon is also evident in hematopoietic stem cells (HSCs). HSCs, which

are one of the most well-characterised populations of somatic stem cells, are responsible for maintaining blood cell formation by self-renewal and multilineage differentiation in the bone marrow, fetal liver and thymus. Rat bone marrow-derived HSCs are transformed *in vitro* to hepatocyte-like cells following treatment with high concentrations of hepatocyte growth factor (Oh *et al.*, 2000). More interestingly, two independent research groups demonstrated the emergence of hepatocytes from donor bone marrow cells following transplantation into lethally irradiated rat (Petersen *et al.*, 1999) and mouse (Lagasse *et al.*, 2000) recipients. The emergence of hepatocytes positive for dipeptidyl peptidase (DDP) IV in the liver of DDPIV-negative female rats rescued by bone marrow transplantation from DDPIV-positive male rats was confirmed by histochemical analysis using a Y-chromosome-specific DNA probe and an antibody against DDPIV. In mice, regeneration of donor-derived hepatocytes was detected in recipient fumarylacetoacetate hydrolase (FAH)-deficient mice following transplantation of whole bone marrow cells, and by intravenous injection of enriched bone marrow cells carrying surface markers specific to HSCs isolated from ROSA26 transgenic mice. These mice also showed significant improvements in FAH associated symptoms.

Thus, somatic stem cells, including those from neural and hematopoietic lineages, retain the potential to transdifferentiate into pluripotent stem cells by extrinsic induction signals (Fig. 1). Adult stem cells therefore have properties that are more closely related to those of embryonic stem cells than previously imagined.

Nuclear reprogramming of somatic cells via nuclear transfer

The transfer of somatic cell nuclei into unfertilized oocytes is one possible approach towards generating totipotent cells classically known as cloned embryos. The production of cloned animals was first achieved more than 30 years ago in amphibians. Cloned frogs were generated by nuclear transfer of the intestinal endoderm of feeding tadpoles into activated enucleated eggs (Gurdon, 1962). Vital to this process was the successful reprogramming of the somatic cell nucleus by the egg cytoplasm. More recently, the nuclear reprogramming capacity of oocytes has been demonstrated in mammals by the production of cloned sheep, cows, mice and pigs (Wilmut *et al.*, 1997; Kato *et al.*, 1998; Wakayama *et al.*, 1998; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000) (Fig. 1). Thus, nuclear reprogramming is not an uncommon phenomenon.

Recent technical developments in the transfer of somatic nuclei have provided a new approach for cell-mediated transgenesis, since the nuclei of cultured somatic cells (as for ES cells) can be genetically manipulated *in vitro* before transfer into enucleated oocytes (McCreath *et al.*, 2000; Rideout *et al.*, 2000). Thus, both nucleo-cytoplasmic

hybrids and somatic cell-mediated transgenic animals could be generated using this approach, in mammalian species in which the establishment of ES cells is not currently feasible. One biomedically valuable advantage of this technique is the production of ES cells derived from blastocysts cloned from somatic cells (Munsie *et al.*, 2000; Kawase *et al.*, 2000). The genotype of these ES cells is identical to that of the original somatic cell, and therefore the immune system of the somatic cell donor will be completely tolerant to the ES cell derived from the cloned blastocyst.

In mice cloned from somatic nuclei one effect of reprogramming is alterations in activity of the X-chromosome. X-chromosome inactivation during early embryogenesis coincides with changes in epigenetic modifications, including a delay of DNA replication to the later stages of S phase (Takagi *et al.*, 1982), DNA hypermethylation (Jones, 1999) and histone H4 hypoacetylation (Keohane *et al.*, 1998). An X-chromosome that is inactivated in a female somatic cell is reactivated in cloned preimplantation mouse embryos (Eggan *et al.*, 2000), indicating that epigenetic reprogramming has occurred in the cloned cells. Other features of cloning include the extension of telomeres in cloned calves, when compared to cells from age-matched, noncloned animals (Lanza *et al.*, 2000). Indeed, telomere shortening was not evident even after six rounds of sequential cloning in mice (Wakayama *et al.*, 2000). Telomere extension, which normally occurs during germline development, would therefore appear to be tightly regulated in cloned animals, although the mechanism by which this occurs is not understood.

Highly differentiated somatic cells are normally incapable of transformation to pluripotent stem cells by extrinsic induction. The successful production of cloned animals from such cells, however, demonstrates that the mammalian unfertilized oocyte contains powerful factors that are capable of reprogramming somatic nuclei from a fully differentiated state to a toti-/pluripotent state.

Nuclear reprogramming of somatic cells by cell hybridization

Similar to the production of cloned animals from somatic nuclear transfer, the epigenetic state of a somatic nucleus can be heritably reprogrammed to that of a pluripotent stem cell by hybridization of the two cell types (Fig. 2A). In cell hybrids generated by the fusion of gonadal PGC derived EG cells and thymic lymphocytes (Tada *et al.*, 1997), the methylation of imprinted genes, non-imprinted genes and repetitive sequences scattered throughout the genome is erased. In addition, tissue-specific gene expression is lost and the somatic nuclei acquire pluripotent stem cell-like properties, as shown by their competence to differentiate into the three primary germ layers of the developing embryo. Hybridization of thymic lymphocytes to ES cells also leads to acquisition of pluripotent competence to differentiate into various tissues (Tada *et al.*, in preparation).

(Fig. 2C). Thus, both types of pluripotent stem cell retain a common capacity to reprogram somatic nuclei through the action of transacting factors. It is notable, however, that the allele-specific methylation pattern associated with many imprinted genes is maintained in the somatic nuclei of ES hybrid cells. This finding differs significantly from the dominant effect of an EG cell on a somatic nucleus, and indicates that “embryonic type” and “germ cell type” reprogramming occurs in ES and EG hybrid cells, respectively. When ES cells are hybridized with thymic lymphocytes carrying an inactive *Oct4-GFP* transgene, which is an ideal marker for the identification of toti-/pluripotent stem cells (Yeom *et al.*, 1996; Yoshimizu *et al.*, 1999), the *Oct4* gene is reactivated within 48 hours (Fig. 2B). This time-lag suggests that DNA replication and cell division may be a central requirement for induction of reactivation. Thus, nuclear reprogramming of somatic cells can be induced not only by factors present in oocyte cytoplasm, but also by transacting factors derived from ES and EG cells.

Extrinsic and intrinsic factors for producing the toti-/pluripotent stem cell

Toti-/pluripotent stem cells are capable of differentiating into a variety of cell and tissue types essential for support of body formation. However, the different toti-/pluripotent stem cells, which possess similar properties to each other, are likely to be produced in different ways reflecting their different cellular origins. ES and EG cells, and pluripotent stem cells transdifferentiated from somatic stem cells, can all be transformed by the action of extrinsic signals. Conversely, the nuclear reprogramming of somatic cells is induced by cellular factors intrinsic to the unfertilized oocyte, ES cell and EG cell in cloning and hybridization.

Induction of toti-/pluripotent stem cells by extrinsic signals occurs *in vivo* during mouse germ cell development. In the eggs of *Drosophila melanogaster*, *Caenorhabditis elegans* and *Xenopus laevis*, the germcell determinant, germ plasm (polar plasm), is accumulated as a maternal factor. However, it is believed that mouse eggs do not contain such a factor (Snow and Monk, 1983), suggesting that germ cell and somatic cell lineages are not committed in the early mouse embryo. PGCs are first detected biochemically as alkaline phosphatase-positive cells localized in the extra-embryonic mesodermal region, posterior to the primitive streak of the E7.25 embryo (Ginsburg *et al.*, 1990). It is largely unknown how and when the PGCs are initially determined in the implantation embryo. However, transgenic mice deficient for the *Bmp4* gene, whose expression is first detected in the extraembryonic ectoderm next to the epiblast of E5.5 embryos (Lawson *et al.*, 1999), do not form PGCs. It is therefore likely that some signals, including BMP4, secreted from the extraembryonic ectoderm induce the formation of PGC precursors in the adjacent embryonic ectoderm. This hypothesis is supported by recent *in vitro* primary cul-

Fig. 2. Nuclear reprogramming of somatic nuclei by cell hybridization with ES cells. (A) Experimental strategy for generating ES x thymocyte hybrid cells and for producing chimeras by blastocyst injection. (B) Reactivation of the *Oct4-GFP* reporter gene, which is a marker for identification of pluripotent stem cells, in ES x thymocyte hybrid cells. *Oct4-GFP* repressed in thymocytes is expressed in all ES hybrid cells. (C) Pluripotential competence of ES x thymocyte hybrid cells as shown by contribution to the three primary embryonic germ layers in E7.5 chimeras. The hybrid cell derivatives are detected as scattered blue cells by X-gal staining in a semithin section of chimeric embryo.

ture experiments on explanted embryos at the prestreak stage (Yoshimizu *et al.*, 2001). Epiblasts dissected from E5.5 embryos, combined with extraembryonic ectoderm tissue, gave rise to clusters of PGC-like cells positive for alkaline phosphatase activity, whereas such cells were not detected in cultured E5.5 epiblasts without extraembryonic ectoderm. Therefore, differentiation of the primary epiblast cells into PGCs, the precursor cells of totipotent germ cells, is triggered by extrinsic factors secreted from the extraembryonic ectoderm.

Blastocysts, which are composed of trophoblast cells (extraembryonic tissue precursors) and pluripotent ICM cells (embryonic tissue precursors), develop into an egg-cylinder stage embryo shortly after implantation. While the ICM cells of preimplantation embryos are capable of transforming into ES cells, the epiblast cells of the early postimplantation embryo are not. The derivation process of ES cells from ICM cells is not fully understood. It is known, however, that supplementary signals added either directly to the medium (leukemia inhibitory factor (LIF)) or derived from fibroblast feeder cells, are required for successful ES cell

derivation. Additional signals are also required for the successful derivation of mouse EG cells, including fibroblast growth factor (FGF) and LIF (Matsui *et al.*, 1992; Resnick *et al.*, 1992). In the transdifferentiation of neural stem cells, extrinsic FGF and epidermal growth factor (EGF), supplied in the culture medium, stimulate rapid proliferation *in vitro* (Doetsch *et al.*, 1999; Johansson *et al.*, 1999; Clarke *et al.*, 2000). Thus, extrinsic inductive factors play key roles in restoring and maintaining the pluripotent stem cell properties on cells derived from PGCs and the stem cells of adult organs, which are relatively undifferentiated in comparison to typical somatic cells. It is, however, not evident that the same factors are involved in their maintenance *in vivo*.

Stimulation by extrinsic factors does not allow the acquisition of pluripotent stem cell properties on considerably differentiated somatic cells. Toti-/pluripotent stem cells with somatic nuclei can be produced only via nuclear reprogramming using the nuclear transplantation technique discussed above (Wilmut *et al.*, 1997; Kato *et al.*, 1998; Wakayama *et al.*, 1998; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000) and *in vitro* cell hybridization between pluripotent

stem cells and somatic cells (Tada *et al.*, 1997; Tada *et al.*, in preparation). Thus, intrinsic factors stored in the host toti-/pluripotential cells are required for remodeling the epigenetic modifications of the somatic nuclei.

Regulation of epigenetic modification

The epigenetic status of stem cells and adult somatic cells from different developmental stages is modified to that of a toti-/pluripotential stem cell, whether stimulated by extrinsic or intrinsic factors. Recent developments concerning epigenetic modifications reveal that DNA methylation, histone acetylation and chromatin remodeling are intimately linked to regulate gene-specific transcriptional activation and repression.

DNA methylation, which is one of the most well-characterized epigenetic modifications associated with transcriptional repression, and is heritable through cell divisions, occurs at the 5'-cytosine residue of CpG dinucleotides in vertebrate genomes (Boyes and Bird, 1991). In mammals, a global erasure mechanism shortly after fertilization results in very low levels of methylation, which is maintained through preimplantation development. After implantation, genome-wide remethylation occurs by a wave of *de novo* methyltransferase activity in the somatic cell lineages (Monk *et al.*, 1987; Razin and Kafri, 1994). In the germ cell lineage, a second round of erasure demethylates the vast majority of methylated sites prior to meiosis, after the PGCs have colonized the genital ridges (Kafri *et al.*, 1992; Brandeis *et al.*, 1993). The methylation event in postimplantation embryos and the demethylation event in gonadal PGCs are accompanied by inactivation and reactivation of one X chromosome, respectively, in a normal female cell (Monk and Harper, 1979; Monk and McLaren, 1981). This is accompanied by the establishment and switching of genomic imprints, which regulate the parental-origin-specific methylation and repression of imprinted genes (Brandeis *et al.*, 1993; Szabo and Mann, 1995; Tada *et al.*, 1998; Obata *et al.*, 1998; Tada *et al.*, 2000). Stable inheritance of methylation patterns from parental DNA to daughter DNA is due to the abundant enzymatic activity of the maintenance methyltransferase (*Dnmt1*). This enzyme preferentially recognizes hemimethylated CpG residues and faithfully recruits methylation to newly synthesized DNA (Bestor, 1988). The embryonic lethal phenotype found in mice deficient for *Dnmt1* clearly demonstrates that methylation is crucial for appropriate regulation of gene expression, most likely by altering the accessibility of transcriptional machinery to the genes (Li *et al.*, 1992, 1993; Howell *et al.*, 2001). However, it is likely that additional methyltransferase enzymes, in conjunction with *Dnmt1*, control global methylation patterns since some residual *de novo* methylation is seen in *Dnmt1* deficient ES cells. Indeed, two further enzymes, *Dnmt3a* and *Dnmt3b*, have been identified recently, both of which carry a methyltransferase domain (Okano *et al.*,

1998). Disruption of these genes resulted in embryonic lethality even though the activity of *Dnmt1* was not affected (Okano *et al.*, 1999). In the dynamics of methylation, how demethylation is regulated remains unknown. To form a stable inactive chromatin structure for gene repression, the methylated-DNA-binding protein complex, MeCP1, and the MeCP2 protein, are required. Both of these contain a methyl-CpG-binding domain (MBD) (Nan *et al.*, 1993). MeCP2, which has a high affinity for single symmetrically methylated CpG dinucleotides, plays an important role since high degree chimeras with *MeCP2*-deficient ES cells are embryonic lethal between E8.5 and E12.5 (Tate *et al.*, 1996). Interestingly, unlike the *Dnmt1*-deficient mice, these chimeras did not show biallelic expression of imprinted genes or ectopic activation of the *Xist* (X inactive-specific transcript) gene in male ES cells (Li *et al.*, 1993; Beard *et al.*, 1995), suggesting that other MBD-related genes play redundant roles. To date, four genes have been identified in mice and humans which carry MBD related sequences, *MBD1*, *MBD2*, *MBD3* and *MBD4*. *MBD1*, *MBD2* and *MBD4* bind specifically to methylated DNA *in vitro* and *in vivo* (Cross *et al.*, 1997; Hendrich and Bird, 1998). Distinctive functions for *MBD2*, which is a component of the MeCP1 protein complex (Ng *et al.*, 1999) and the closely related *MBD3* protein, which is a component of the Mi-2/NuRD co-repressor complex (Tong *et al.*, 1998; Zhang *et al.*, 1998; Wade *et al.*, 1999), have been revealed by gene disruption experiments. *Mbd2*-deficient mice were viable and fertile, whereas *Mbd3*-deficient mice died before E8.5, suggesting that *MBD3* is a key component of Mi-2/NuRD, while MeCP1 is recruited to DNA by factors other than *MBD2* (Hendrich *et al.*, 2001).

Important progress has been made in understanding the mechanism of chromatin remodelling by interactions between DNA methylation and histone deacetylation. In general, histone acetylation, through the action of histone acetylases (HATs), correlates with gene activation. Conversely deacetylation, *via* histone deacetylases (HDACs), correlates with gene silencing (Turner, 1993; Wolffe, 1996; Fry and Peterson, 2001). The histone acetylation status of conserved lysine residues in the amino-terminal domains of histones H3, H4, H2A and H2B directly contributes to transcriptional regulation. However, the molecular mechanisms underlying the effects of histone acetylation and deacetylation on transcription are not fully understood. Acetylated amino-terminal domains extending from the surface of the nucleosome contribute to nucleosome destabilisation (Bauer *et al.*, 1994) and disruption of higher-order compaction of nucleosomal arrays (Tse *et al.*, 1998) without altering histone-histone interactions and disruption of nucleosome assembly. A remarkable hallmark in the field of gene regulation is that MeCP2 recruits a corepressor complex containing HDACs and mSin3A (Nan *et al.*, 1998; Jones *et al.*, 1998). Thus, two mechanisms of gene repression, involving methylated chromatin and de-acetylation of core histones,

are bridged through the direct or indirect mediation of mSin3A. Similarly, cooperation of HDACs and MBD1, MBD2 and MBD3 may also be involved in the mechanism of gene silencing (Newell-Price *et al.*, 2000; Ballestar and Wolffe, 2001). The HDAC-dependent pathway is at least in part responsible for transcriptional repression.

A recent study shows a link between chromatin remodeling, methylation and gene expression in mammals. Mutations in the *ATRX* gene, which contains a highly conserved plant homeodomain-like region and is a member of the SWI/SNF2 helicase/ATPase family, give rise to alterations in methylation profiles at the highly repeated sequences, rDNA arrays, a Y-specific satellite and subtelomeric repeats (Gibbons *et al.*, 2000). Thus, genome wide demethylation may occur as a consequence of chromatin remodeling during mammalian development. In yeast, the SWI2/SNF2 family of ATPases, which are members of a large subfamily of the DEAD/H superfamily 2 of nucleic acid-stimulated ATPases and DNA helicases (Carlson and Laurent, 1994), consists of more than 35 members which function in gene transcription, DNA repair and recombination, chromosome segregation and cell cycle progression (Pollard and Peterson, 1998). ATP-dependent chromatin-remodeling complexes that harbor SWI2/SNF2 family members have been termed dACF, dNURF, dCHRAC and Brahma in *Drosophila*, xMi-2 and xACF in frog and hSWI/SNF, hNURD and hRSF in human (Fry and Peterson, 2001). In mammals, the SWI/SNF-like complexes consist of at least two groups distinguished by their ATPase subunits, BRG1 and BRM. Both human BRG1 and BRM show similar ATPase activity, as shown by their activity on mononucleosomes and nucleosomal arrays using purified proteins (Phelan *et al.*, 1999). However, distinct roles are evident between the mouse SWI/SNF-like complexes, BRG1-related and BRM-related, as shown by deletion of these genes (Reyes *et al.*, 1998; Bultman *et al.*, 2000). *Brm*-deficient mice are viable, whereas *Brg1*-deficient mice die during preimplantation development, indicating that *Brg1* plays an essential role in the preimplantation embryo. The SWI/SNF-like complexes have chromatin remodeling activity, whereby the alteration of nucleosome conformation utilises energy derived from ATP hydrolysis, possibly by influencing the degree of twist on the nucleosomal DNA. To generate a stable remodeled nucleosomal state through DNA untwisting should require continuous ATP hydrolysis, since the remodeled state should be unstable and rapidly revert back to the original state (Gavin *et al.*, 2001). Analysis of the localization and retention of the yeast SWI/SNF complex to a promoter in a purified system provides some evidence for this. The stability of SWI/SNF binding to the promoter element was enhanced in the presence of nucleosomes acetylated by either SAGA or NuA4 HAT complexes, when compared to binding in the presence of an unmodified nucleosome array (Hassan *et al.*, 2001). This stable promoter occupancy by SWI/SNF was maintained even after the HAT complexes

were dissociated. This implies that the activities of activator-recruited HAT complexes and activator-recruited ATP-dependent chromatin remodeling complexes are functionally linked. Properly ordered recruitment of these complexes is required to remodel the nucleosome conformation of a promoter region in a stable manner.

Thus, transcriptional activator and repressor proteins are able to interact with each other, bind to the promoter regions of genes, and recruit numerous co-activator and co-repressor proteins including SWI/SNF-like ATPases, HATs, HDACs and methylation-related proteins to regulate the accessibility of components of the general transcriptional machinery. It is, however, still unclear which of these interactions are crucial, and whether the order of recruitment of these factors is critical for appropriate chromatin conformation remodeling, in the regulation of transcriptional activation and repression.

What is the epigenetic status of toti-/pluripotent stem cell nuclei?

The epigenetic status of adult stem cells and nuclei of considerably differentiated somatic cells can be 'reverted' to that of toti-/pluripotent stem cells by extrinsic and intrinsic factors, respectively. Mouse ES cells, which have a robust capacity to proliferate, are hypomethylated in comparison to considerably differentiated somatic cells, even though such cells possess *de novo* methylation activity as shown by the stable methylation of Moloney murine leukemia virus sequences after infection of EC cells (Niwa *et al.*, 1983). However, little is known about the epigenetic status of toti-/pluripotent stem cells. One approach towards understanding this question is to address the molecular mechanisms of somatic cell nuclear reprogramming in the process of animal cloning.

The epigenetic status of endogenous nuclei in eggs should indicate what we might expect to find in cloned somatic nuclei following transplantation. One general phenomenon is that of transcriptional repression in zygotes during early developmental stages. In *Xenopus laevis*, prior to the beginning of zygotic transcription at the midblastula transition (MBT), the genome is replicated rapidly and transcription is repressed. To this end there is an important contribution of the maternal DNA methyltransferase (*xDnmt1*) enzyme, demonstrated by precocious activation of marker genes following depletion of *xDnmt1* by antisense RNA (Stancheva and Meehan, 2000). The level of 5-methylcytosine in the *Xenopus* embryo gradually decreases prior to the MBT (Stancheva and Meehan, 2000). Thus, methylation may be involved in maintaining transcriptional repression from cloned somatic nuclei that have been reprogrammed by egg factors. Another mechanism that could be involved in regulating the prevention of zygotic expression is removal of the TATA binding protein (TBP) from the paternal nucleus by the maternal activity of the nucleosome-dependent

ATPase, ISWI. ISWI is a member of the SWI2/SNF2 superfamily. When nuclei from *Xenopus* XTC-2 epithelial cells were briefly incubated in *Xenopus* egg extract, they were remodeled and showed the ISWI-mediated loss of TBP, which is a key component of the basal transcriptional complex (Kikyo *et al.*, 2000). What is important is that ISWI may function as a chromatin remodeler in the process of nuclear reprogramming of cloned somatic nuclei. Disappearance of the TBP removal activity by twofold dilution of the extract implies that this event may occur as an initial step in the process of nuclear reprogramming. However, the overall chromatin conformation of somatic nuclei reprogrammed in eggs is still largely unknown. To address this fully it is essential to know more about the possible factors and events that are involved in the reprogramming process.

In mammals, the timing of the beginning of zygotic expression varies among species, from the 1-2-cell stage in mice, the 4-cell stage in pigs and the 8-16-cell stage in sheep and cows. In all of these species, the successful production of cloned animals by nuclear transplantation implies that DNA synthesis through the cell cycle may not be essential for at least an initial step in the nuclear reprogramming of somatic cells. However, this does not rule out the involvement of cell cycle-related factors in this event. It is of interest that the production of cloned cows succeeds at a relatively high frequency compared to the cloning of mice (Kato *et al.*, 1998; Wakayama *et al.*, 1998; Ogura *et al.*, 2000). One possible explanation is that, for full nuclear reprogramming, somatic nuclei have to be exposed to maternal factors for some time prior to the onset of zygotic tran-

scription. Thus, reprogramming would be expected to occur more efficiently in cloned cow and sheep embryos than in cloned mice. In mice, zygotic expression begins from the late 1 cell stage (Latham *et al.*, 1992), while the maternal mRNA pool is dramatically reduced (Worrad *et al.*, 1994). The contents of the egg are therefore dynamically altered between pre- and postzygotic expression. After fertilization, the paternal and maternal genomes are united. The sperm chromatin is decondensed, during which process protamines are replaced with histone H2A and H2B, mediated by the action of nucleoplasmin (Laskey *et al.*, 1993). The maternal pronuclear chromatin remains relatively condensed, and the genomes start to replicate asynchronously (Ferreira and Carmo-Fonseca, 1997). Strikingly, rapid and active demethylation of the paternally derived endogenous *Igf2* gene and the paternal *TKZ751* transgene takes place in mouse zygotes within 4 hours of fertilization, in the absence of DNA replication (Oswald *et al.*, 2000; Mayer *et al.*, 2000). Furthermore, the carboxy-terminal domain of RNA polymerase II, which was predominantly hyperphosphorylated, becomes markedly dephosphorylated within several hours of fertilization (Bellier *et al.*, 1997). Eventually, the major epigenetic status of the two genomes must be functionally equivalent and their chromatin structure must be suitable for the onset of zygotic expression. Studies into the nuclear reprogramming of the paternal genome in the oocyte cytoplasm could, therefore, provide insight into the mechanism of nuclear reprogramming of cloned somatic cells. If so, the chromatin of somatic nuclei should be generally decondensed and demethylated shortly after exposure to the egg cytoplasm

Fig. 3. A model of chromatin remodelling through nuclear reprogramming of somatic cells. In nucleus of a somatic cell, a relatively tight nucleosome structure is formed to precisely regulate transcription of tissue-specific genes. In the nucleus of a pluripotent stem cell, tissue-specific nucleosome formation may be disrupted and a relatively loose nucleosome structure is formed.

(Fig. 3). This is consistent with the finding in *Xenopus* that decondensed chromatin structure could be formed by the erasure of TBP from the somatic nucleus via the action of nucleosome-dependent ATPases (Kikyo *et al.*, 2000). However, we do not yet know when a totipotent cloned nucleus becomes fully established. Other remodeling factors may contribute to further reprogramming of cloned nuclei in *Xenopus* early development.

For acquisition of totipotent competence, reprogramming of the somatic nucleus by oocyte cytoplasmic factors is undoubtedly evidenced by the production of cloned animals. Similar nuclear reprogramming events are demonstrated by cell hybridization experiments between ES/EG cells and thymic lymphocytes (Tada *et al.*, 1997; Tada *et al.*, in preparation). This suggests that nuclear reprogramming factors are not specific to oocyte cytoplasm, and production of these factors from stem cells can be stimulated by extrinsic factors during ES/EG cell derivation. Reprogrammed somatic nuclei in hybrid cells retain similar properties to the host stem cell nuclei, since tissue-specific genes are repressed and both nonimprinted and imprinted (in the case of EG cell fusions) genes become demethylated (Tada *et al.*, 1997). Retroposons, such as the Line-1 repetitive elements, the L1 family, and B2 genes, are actively transcribed by RNA polymerase III in the nuclei of ES and EG cells, and their hybrids with somatic cells (unpublished observations). This has also been observed in mouse blastocysts (Packer *et al.*, 1993) and F9 EC cells (White *et al.*, 1989). These findings allow us to speculate that the chromatin of somatic nuclei may be decondensed following hybridization with ES and EG cells (Fig. 3). Factors facilitating loose chromatin formation could therefore play key functions in the reprogramming of cloned somatic nuclei. The regulatory mechanisms, and key factors, that are responsible for reprogramming of somatic nuclei are only now beginning to be elucidated. Greater understanding of these mechanisms will open new and exciting fields in the areas of both basic science and clinical medicine.

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