Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells

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The resetting of a somatic epigenotype to a totipotential state has been demonstrated by successful animal cloning, via transplantation of somatic nuclei into enucleated oocytes. We have established an experimental system, which reproduces the nuclear reprogramming of somatic cells in vitro by fusing adult thymocytes with embryonic stem (ES) cells. Analysis of the lymphoid-cell-specific V-(D)-J DNA rearrangement of the T cell receptor and immunoglobin genes shows that the ES cells have hybridized with differentiated cells. In these ES cell hybrids, the inactivated X chromosome derived from a female thymocyte adopts some characteristics of an active X chromosome, including early replication timing and unstable *Xist* transcription. We also found that an *Oct4-GFP* transgene, which is normally repressed in thymocytes, is reactivated 48 hr after cell fusion. The pluripotency of the ES-thymocyte hybrid cells is shown in vivo, since they contribute to all three primary germ layers of chimeric embryos. The somatic DNA methylation pattern of the imprinted *H19* and *Igf2r* genes is maintained in these hybrids, unlike hybrids between ES and EG (embryonic germ) cells in which the differential methylation is erased. Thus, ES cells have the capacity to reset certain aspects of the epigenotype of somatic cells to those of ES cells.

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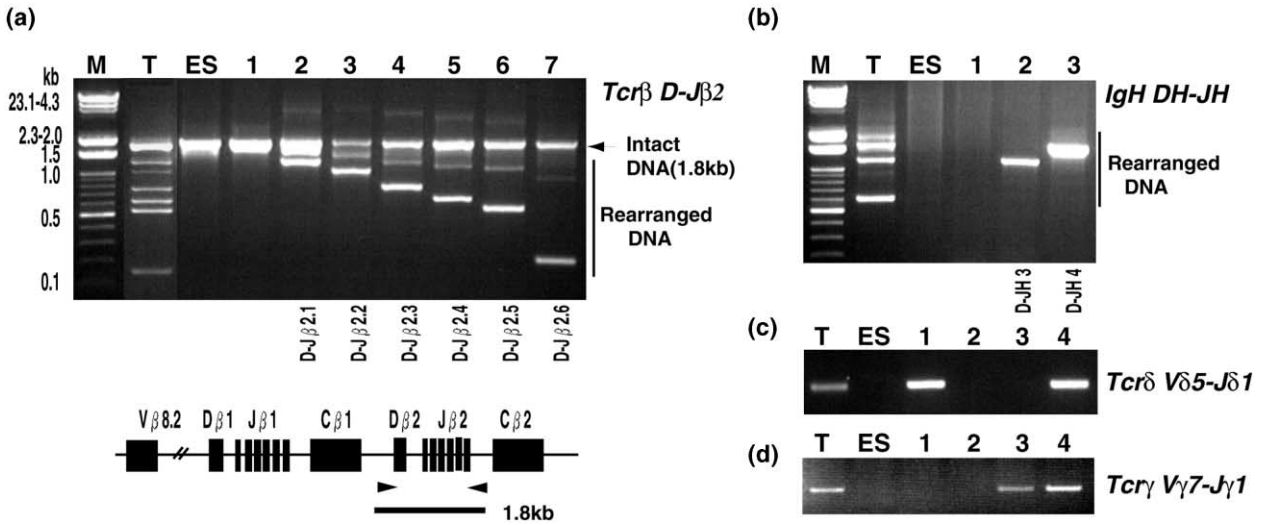
Results and discussion

The capacity for reprogramming somatic nuclei has now been demonstrated by nuclear transplantation to enucleated oocytes in many mammalian species [1]. To reproduce nuclear reprogramming in vitro, we hybridized adult thymocytes with embryonic stem (ES) cells. To ensure that ES cells hybridized with differentiated cells, PCR amplification with four primer sets specific to the D-J region of T cell receptor (*Tcr*)β, the D-J region of immunoglobulin (*Ig*) H, and the V-J region of *Tcr*δ and *Tcr*γ was performed on DNA extracted from ES hybrid cells with thymocytes. DNA rearrangement is one of the clear signs that thymocytes have differentiated into lymphoid cells [2]. In 45% of the hybrid clones, rearrangements specific to *Tcr*β D-Jβ 2.1, -2.2, -2.3, -2.4, -2.5, or -2.6 were found (Figure 1a). Similarly, rearrangements of the *IgH* D-J region (Figure 1b) and the V-J region of *Tcr*δ (Figure 1c) and *Tcr*γ (Figure 1d) were found in some clones. In total, out of 31 ES hybrid clones, 17 (55%) carried at least one of the rearrangements under investigation. Thus, in these cases, the ES cells had hybridized with thymocyte nuclei after differentiation to lymphoid cells.

To analyze X chromosome activity, we studied the well-characterized phenomenon of X chromosome replication asynchrony using a replication-banding technique (Figure 2a) [3]. After continuous incorporation of BrdU through the second half of S phase and acridine orange staining, the active X chromosome and the autosomes are seen as banded red and green elements. The inactive X chromosome is uniformly dull red in a female somatic cell (Figure 2b), due to delayed replication. In 6 clones of hybrid cells between XY male ES cells and XX female thymocytes, all 32 cells karyotyped (4n = 80) carried 3 synchronously replicating X chromosomes (Figure 2c). Consistent with this, *Xist* (inactive X-specific transcript) RNA was unstably accumulated (spotted) on three X chromosomes in two ES hybrid cell lines examined by RNA FISH (fluorescent in situ hybridization) (Figure 2d). *Xist* RNA accumulation was also unstable on the active X chromosome of a male ES cell, but was stable on the inactive X chromosome of a female thymocyte (painting signal). Thus, the inactive X chromosome derived from the somatic nucleus adopts several characteristics of an active X chromosome after hybridization, namely, replication and a pattern of *Xist* expression found in undifferentiated cells.

To visualize reprogramming of the somatic cell nuclei, we used a mouse strain carrying an *Oct4-GFP* transgene

Figure 1



DNA rearrangement of *Tcr*β, *Tcrδ, Tcr*γ, and *IgH* genes derived from thymocytes in ES hybrid cells. **(a)** D-J region of *Tcr*β, **(b)** D-J region of *IgH*, **(c)** V-J region of *Tcr*δ, and **(d)** V-J region of *Tcr*γ. PCR analysis detects intact and rearranged DNA of Dβ2-Jβ2 of *Tcr*β in (a). The intact DNA is visualized as a 1.8-kb band by PCR amplification with primers (shown between arrowheads in the genomic map). T, thymocytes from (Rosa26 × Oct4-GFP)F1 mice; ES, ES cells; M, marker mixture of λ/HindIII DNA and 100-bp ladder DNA.

Figure 2

Reactivation of an X chromosome derived from thymocytes in ES hybrid cells. **(a)** R-banding analysis of X chromosome replication timing in ES hybrid cells. In ES hybrid cells, three X chromosomes (two X chromosomes from a female thymocyte and one X chromosome from a male ES cell) are detected as red and green elements, showing that they are active. Three synchronously replicating X chromosomes in (a) are magnified in **(c)** (arrowheads). An X chromosome in a female somatic cell (arrow in **[b]**) and a Y chromosome (in [a]) are stained uniformly red, indicating that they are inactive. **(d)** *Xist* RNA is detected as a spotted red signal on an active X chromosome of a male ES cell, whereas *Xist* RNA is coating an inactivated X chromosome of a female thymocyte, as shown by a large red signal. Three spotted red signals per nucleus are detected in two ES hybrid cell lines (ES × T1 and ES × T2) examined.

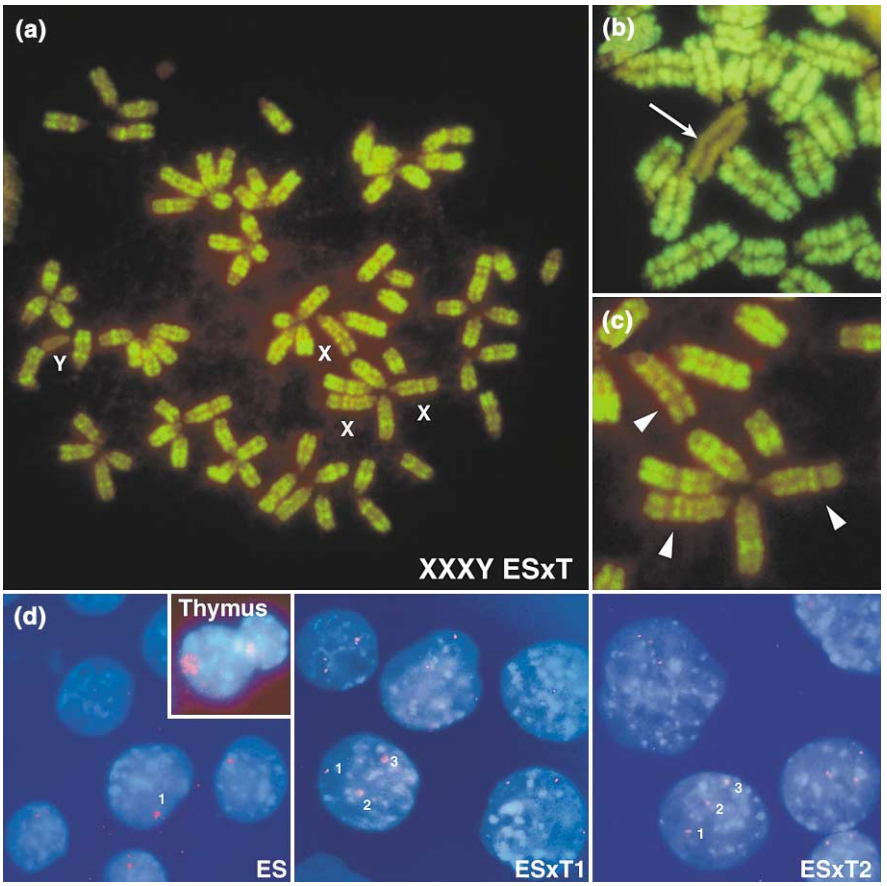
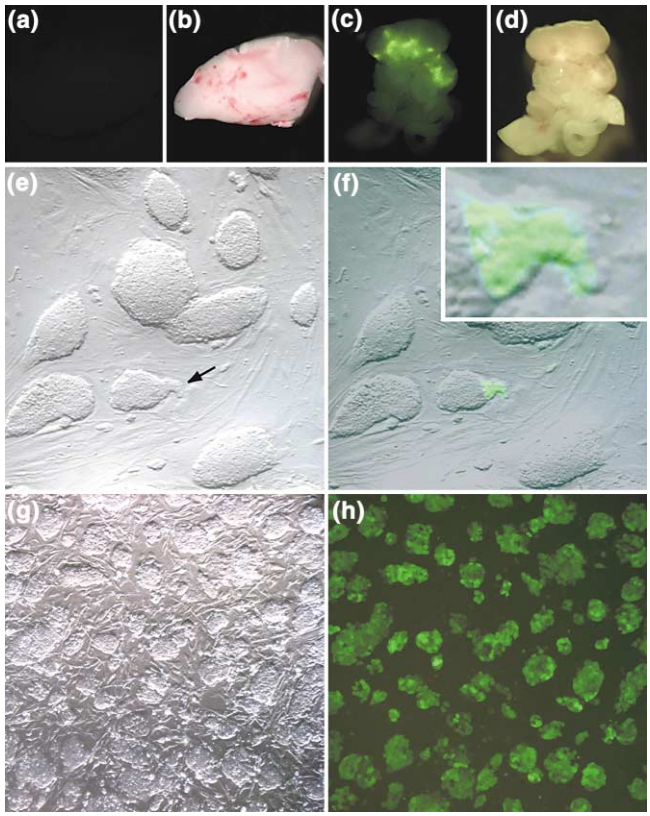


Figure 3



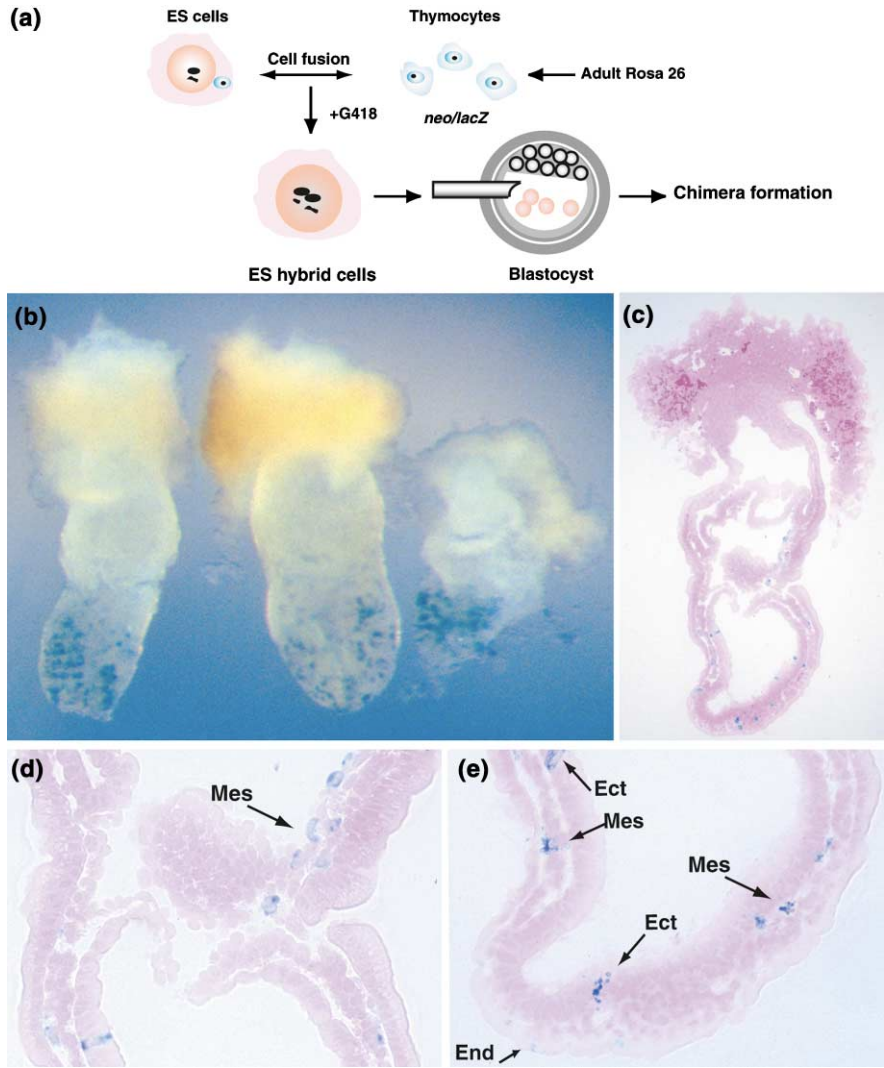
Reactivation of *Oct4-GFP* in ES hybrid cells. GFP-fluorescent images and bright field images of the **(a,b)** thymus the **(c,d)** ovary from (Rosa26 × Oct4-GFP)F1 mice used for the production of ES hybrid cells. **(e)** A bright field image of **(f)** (the arrow shows the GFP-positive colony). (f) A small GFP-positive colony alongside nonexpressing ES cell colonies on the second day after fusion. The positive colony is shown under higher magnification in the inset. **(g)** A bright field image of **(h)**. (h) GFP-positive cells expanded from a colony after G418 selection.

(Figure 3). *Oct4* expression is unique to germ cells, preimplantation embryos, and the epiblast of early postimplantation embryos. Therefore, the activity of *Oct4* provides an ideal marker for the identification of toti- and/or pluripotent cells. The *Oct4-GFP* expression pattern is comparable to the endogenous *Oct4* expression pattern [4]. To ensure this, transgenic thymuses and ovaries were observed. GFP was not detected in the thymocytes, but was detected in the growing oocytes (Figure 3a–d). Thymocytes from the *Oct4-GFP* transgenic mice were hybridized with ES cells, cultured without selection, and observed every 12 hr for GFP expression. A GFP-positive colony, consisting of 16 cells located at the edge of a larger nonexpressing colony, was first detected after 48 hr (Figure 3e,f). Subsequently, several additional GFP-positive colonies were observed on the same culture plate, prior to confluence. No GFP-positive cells were found in unfused thymocytes cultured under the same conditions. To address whether reprogramming of the somatic nuclei occurred in all ES hybrid cells, thymocytes from (Rosa26 × Oct4-GFP)F1 mice, which are resistant to G418 selection, were used. Following selection, 36 out of 37 (97%) clones that were obtained expressed GFP. This expression was stably maintained through several passages of subculture (Figure 3g,h), demonstrating reprogramming of the thymocyte nuclei in the majority of ES hybrid cells.

To investigate developmental competence, the hybrid cells were microinjected into normal diploid blastocysts (Figure 4a). We used one hybrid clone with a (differentiated) thymocyte from (Rosa26 × Oct4-GFP)F1 mice and one with a thymocyte from Rosa26 mice. Staining for β-galactosidase activity showed the relative contribution of hybrid cells in each chimera, which was expected to be poor due to severe loss of tetraploid cells in chimeras of diploid and tetraploid embryos [5]. Eight out of 20 E7.5 embryos were positive, showing restricted contribution of the hybrid cells (Figure 4b,c). Detailed analysis revealed the presence of hybrid cell derivatives in the embryonic ectoderm, embryonic mesoderm, and visceral endoderm (Figure 4d,e). Thus, the ES hybrid cells possess the developmental potential to differentiate into the three primary germ layers of early postimplantation embryos, although we were not able to determine whether tissue-specific markers are also appropriately expressed from the thymocyte genome.

We next investigated whether nuclear reprogramming of thymocytes also affected methylation at imprinted loci. The maternally expressed *H19* locus contains a paternally methylated region upstream of the gene, thought to hold the primary methylation imprint [6]. Following digestion with BamHI and the methylation-sensitive restriction enzyme HhaI, a 3.8-kb SacI probe detected paternally methylated 10-kb and 2.7-kb fragments and maternally unmethylated 7.0-kb and 1.8-kb fragments in DNA from both thymocytes and ES cells. The same pattern was seen in the hybrid clones, with no difference in relative intensity (RI) between the methylated (RI = 0.60) and unmethylated (RI = 0.40) bands (Figure 5a). Similar results were obtained using the BamHI probe, which identified a paternally methylated fragment at 2.7 kb and maternally unmethylated fragments at 1.8 and 0.8 kb. The methylated (RI = 0.55) and unmethylated (RI = 0.45) bands are similarly detected in all samples (Figure 5a). Another well-characterized primary methylation imprint is an intronic CpG island of the maternally expressed *Igf2r* gene. This region, however, is only methylated on the expressed allele [7]. Following digestion with PvuII and the methylation-sensitive restriction enzyme MluI, a 330-bp *Igf2r* CpG island probe detected a 2.9-kb maternally derived methylated fragment and a 2.0-kb paternally derived unmethylated fragment in DNA from both thymocytes and ES cells (Figure 5b). The same pattern

Figure 4



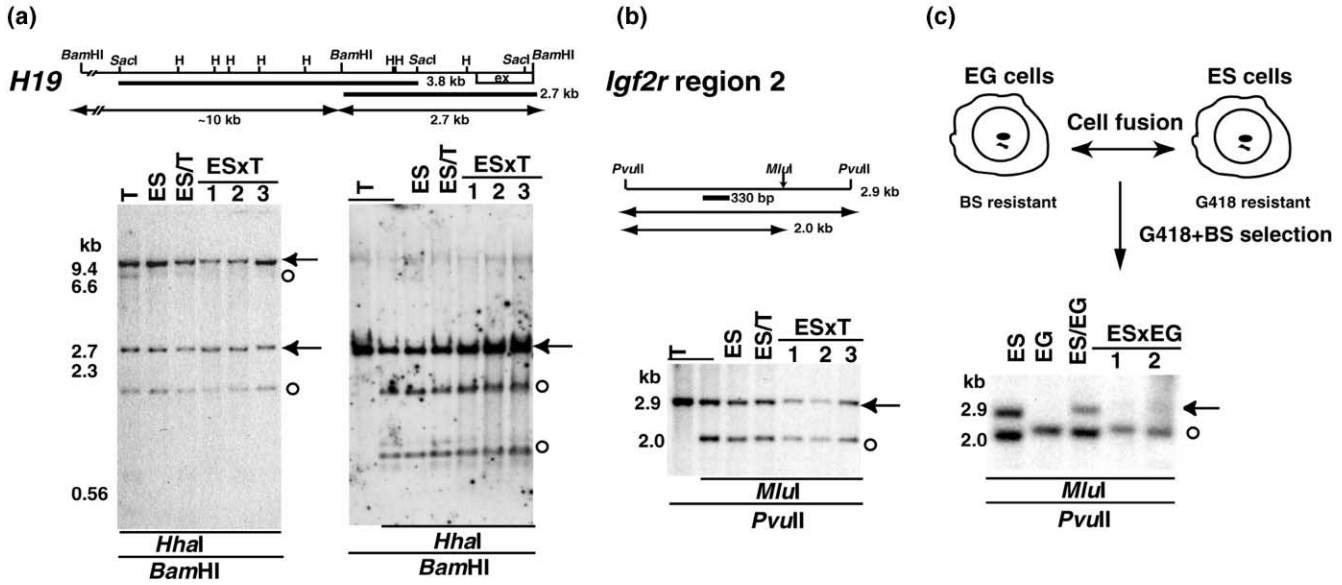
The developmental potential of ES hybrid cells in vivo. **(a)** An experimental scheme for generating ES hybrid cells and making chimeric embryos. **(b)** E7.5 chimeric embryos with ES hybrid cells. The contribution of hybrid cells is shown as blue cells with X-gal staining. **(c)** A representative longitudinal thin section of an E7.5 chimeric embryo. **(d,e)** Thin sections of chimeric embryos at higher magnification. Ect, ectoderm; Mes, mesoderm; End, endoderm.

was seen in DNA from the hybrid clones, with no difference between the RI of the methylated (RI = 0.55) and unmethylated (RI = 0.45) bands. These findings demonstrate that the primary methylation of both the *H19* upstream region and the *Igf2r* intronic region in the thymocyte genome is not affected after hybridization with ES cells.

The finding described above differs from our previous observations in hybrid clones between thymocytes and EG cells derived from gonadal PGCs of E12.5 mouse embryos [8], in which the maternal-specific methylation of *Igf2r* was erased. Maintenance of the somatic methylation pattern in ES hybrid cells suggests that the regulatory mechanism controlling DNA methylation of imprinted genes differs between ES and EG cells. To investigate this, we produced hybrids between ES and EG cells (Figure 5c). Maternal allele-specific methylation of *Igf2r* was detected in ES cells, but not in EG cells, and was detected at a ratio of approximately 1:3 (methylated/RI = 0.27, unmethylated/RI = 0.73) in a 1:1 control mixture of ES and EG cell DNA. In ES × EG hybrids, the methylated band was lost, demonstrating that the demethylation activity present in EG cells is dominant to the methylation imprint maintenance seen in ES cells.

We demonstrate an in vitro system for reprogramming somatic nuclei by generating ES × somatic cell hybrids. We used these hybrids to examine X chromosome replication timing, *Xist* RNA accumulation, exogenous *Oct4* gene expression, developmental potential, and DNA methylation of the imprinted *H19* and *Igf2r* genes. In female somatic cells, one of two X chromosomes is randomly inactivated for dosage compensation of X-linked genes. X chromosome inactivation occurs during early cell differentiation and induces epigenetic modifications, including

Figure 5



DNA methylation of the *H19* and *Igf2r* genes in ES hybrid and ES × EG hybrid cells. **(a)** *H19*; DNA was digested with BamHI and the methylation-sensitive restriction enzyme HhaI and was hybridized with 3.8-kb SacI and 2.7-kb BamHI probes (black bars in the genomic map). Three ES hybrid clones between ES cells and Rosa26 thymocytes, and their parental cells, were analyzed. **(b)** *Igf2r*; DNA was digested with PvuII and the methylation-sensitive restriction enzyme MluI and was hybridized with a 330-bp *Igf2r* intronic CpG island-specific probe (black bar in the genomic map). Three ES hybrid clones between ES cells and Rosa26 thymocytes, and their parental cells, were analyzed. **(c)** *Igf2r*; DNA methylation of the same MluI site was analyzed in two ES × EG hybrid clones. Arrows indicate methylated DNA fragments, and open circles mark unmethylated DNA fragments. The experimental scheme is summarized. T, thymocytes; ES/T, 1:1 mixture of ES and thymocyte DNA; ES/EG, 1:1 mixture of ES and EG DNA.

the delay of DNA replication to late S phase, DNA hypermethylation, and histone H4 hypoacetylation. In cloned embryos made by nuclear transplantation of somatic nuclei into oocytes, the inactive X chromosome of a female somatic cell is reactivated [9]. Thus, both X chromosomes become active, providing an indicator for the occurrence of nuclear reprogramming. Changes in replication timing and *Xist* RNA accumulation on a somatic X chromosome in ES hybrid cells indicated that somatic nuclei are reprogrammed after cell fusion. This was supported by our analysis of *Oct4-GFP* expression in the hybrids. The *Oct4-GFP* transgene, which was repressed in thymocytes prior to cell fusion, was reactivated in the ES hybrid cells. The third, and most conclusive, piece of evidence that the somatic nuclei were reprogrammed in the ES hybrids came from their ability to contribute to the formation of the endoderm, mesoderm, and ectoderm during early development of chimeric embryos. We conclude that ES cells have the capacity to reprogram at least some aspects of somatic nuclei in vitro, providing an experimental system that can be manipulated to investigate the molecular mechanisms involved.

In contrast to the reprogramming activities discussed above, the somatic differential methylation pattern of *H19* and *Igf2r* remained unchanged after thymocyte fusion with ES cells. A similar situation was found in EC (embryonic carcinoma)-thymocyte hybrid cells [10]. However, this allele-specific methylation represents a primary imprint, which is normally retained through postfertilization development, but not through germ cell development [6, 7]. Indeed, in EG-thymocyte hybrid cells, the somatic methylation pattern of several imprinted genes, including *Igf2r*, was disrupted, and both alleles became undermethylated [8]. Taken together, both ES and EG cells appear to retain similar cellular factors that are able to reprogram the epigenetic status of a somatic nucleus, making it competent for embryonic development. However, unlike EG cells, ES cells are not able to reprogram parental imprints. Methylation analysis of *Igf2r* in ES × EG hybrid cells indicates that EG cells may carry an additional dominant factor involved in more extensive epigenetic reprogramming. Indeed, the properties of ES and EG cells appear to reflect those of their cellular origins. Thus, both ES and EG cells should provide useful materials for identifying factors involved in epigenetic reprogramming and demethylation in early embryonic cells and in gonadal PGCs.

Considering the cloning of animals from somatic nuclei, it is clear that the proportion of clones that survive to adulthood is extremely low. Loss of embryos before implantation may be in part due to the failure of nucleo-

cytoplasmic interactions [11, 12]. Furthermore, many cloned embryos are lost during midgestation and soon after birth. One possible reason for this developmental failure is a lack of sufficient reprogramming of the somatic nuclei. If this were a common phenomenon, we may have predicted that somatic *Oct4-GFP* would not be expressed in 100% of the ES hybrid clones generated in this study, because *Oct4* expression is required for the maintenance of developmental potential [13]. However, we observed stable GFP expression in the majority of ES hybrid clones examined, suggesting that, in this system, successful nuclear reprogramming was the norm. For the *H19* and *Igf2r* genes, the primary methylation imprint from somatic cells was maintained in the ES hybrids, indicating that the epigenetic profile of some genes is not affected by cell fusion. This is supported by the finding that an inactive X chromosome from a somatic cell "remembers" its origin and is nonrandomly chosen for inactivation in the trophectoderm cells of cloned embryos [9]. It is, however, possible that the epigenetic profiles of some key genes responsible for normal development are aberrantly reprogrammed during the cloning process.

The mechanisms involved in epigenetic reprogramming of somatic nuclei, leading to competence for normal embryonic development, remain largely unexplored. It has been shown recently that mutations in the *ATRX* gene, which is a member of the SWI2/SNF2 helicase/ATPase family, give rise to alterations in methylation profiles of highly repeated sequences in mammals [14]. Thus, demethylation may occur as a consequence of chromatin remodeling. It has been suggested that the maternal activity of nucleosome-dependent ATPase ISWI may function as a chromatin remodeler in the process of nuclear reprogramming of cloned somatic cells in frogs [15]. Nuclei of the *Xenopus* XTC-2 epitheial cells incubated briefly in *Xenopus* egg extract are remodeled and lose TBP as a key component of the basal transcriptional complex. The reprogramming activity of ES cells may, therefore, facilitate the formation of loose chromatin, leading to the loss of somatic cell epigenetic memory.

Epigenetic instability of some imprinted genes in murine ES cells suggests that the epigenetic status of human ES cells must be assessed before clinical applications [16]. If the host ES cell chromosomes could be eliminated successfully, ES hybrid cells would provide useful therapeutic tools. Ultimately, epigenetic engineering may be enabled through the use of reprogramming factors, once they have been identified. We could then envisage the production of clonal or tissue-specific stem cells, from adult somatic cells, without the need for a contribution from mammalian embryos. Such technology would have important implications in the production of donor cells for numerous clinical applications involving cell or tissue transplantation.

Supplementary material

Supplementary material including full Materials and methods is available at http://images.cellpress.com/supmat/supmatin.htm.

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