Targeted chromosome elimination from ES-somatic hybrid cells

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To engineer a stem cell genome, we developed a technology for targeted elimination of chromosomes from mouse embryonic stem (ES)–somatic hybrid cells. Here we demonstrate the use of a universal chromosome elimination cassette (CEC) for elimination of a single embryonic stem cell (ESC)-derived chromosome 11 or 12, and also both copies of chromosome 6, which harbor pluripotency-associated genes including *Nanog*. We attribute hybrid-cell pluripotency to the expression of *Nanog* from the reprogrammed somatic-cell nuclei.

ESCs possess nuclear reprogramming capacity capable of conferring pluripotency on somatic cell–derived nuclei through cell fusion in both mouse and man[1,2]. To make tetraploid ES-somatic hybrid cells partially or fully personalized, it is necessary to eliminate the ESC-derived chromosomes once the somatic genome has been reprogrammed. Although genome engineering by Cre-*loxP*–mediated chromosome rearrangements has facilitated genetic studies[3,4], large autosomal deletions and whole-autosome loss were found to be detrimental to ESC survival. Therefore, it had been concluded that elimination of autosomes leads to cell lethality in diploid cells.

To eliminate ESC-derived chromosomes from tetraploid hybrid nuclei, we designed a CEC bearing a fluorescent reporter and drug-resistance gene between oppositely oriented *loxP* sites (**Fig. 1a** and **Supplementary Methods** online). Cre-mediated sister-chromatid recombination in late S and G2 phases of the cell cycle should generate dicentric and nullicentric chromosomes. Such aberrant chromosomes are spontaneously deleted from cells during cell division. To test this hypothesis, we electroporated a plasmid containing a CEC with a *gfp* reporter and a gene conferring puromycin resistance (pCEC-CAG-gfp/IRES.puro-pA) into *Hprt1*-deficient HM-1 ESCs. Out of 57 puromycin-resistant GFP-positive clones, we randomly selected two clones and analyzed them by fluorescence *in situ* hybridization (FISH) using a CEC-specific probe. Both clones retained normal karyotype ($2n = 40$, XY). In one clone, the CEC had integrated into the distal region of chromosome 11 (CEC11; **Fig. 1b**). In the other clone, the CEC was on proximal chromosome 12 (CEC12; **Fig. 1c**). We fused each clone with thymocytes from female ROSA26 mice and selected the hybrid cells on hipoxanthine, aminopterin and thymidine medium. We clonally isolated both CEC11 and CEC12 hybrid cell lines, and each cell line had a full set of chromosomes ($4n = 80$, XXXY; **Supplementary Fig. 1** online).

Next we induced CEC-mediated sister-chromatid recombination by transient Cre expression after pCMV-Cre lipofection. After 10 d of culture without selection with 3–4 rounds of subculture, we sorted $\sim 5.0 \times 10^4$–2.5×10^5 cells by fluorescence-activated cell sorting (FACS; **Supplementary Fig. 1**). After Cre treatment, the GFP-negative population substantially increased in both CEC11 (from 0.8 to 2.2%) and CEC12 (from 3.3 to 10.1%) hybrid cells. Consistent with this, without Cre treatment, all hybrid colonies were GFP-positive, but after Cre treatment, FACS-sorted GFP-negative hybrid cells gave rise to GFP-negative colonies (**Supplementary Fig. 1**).

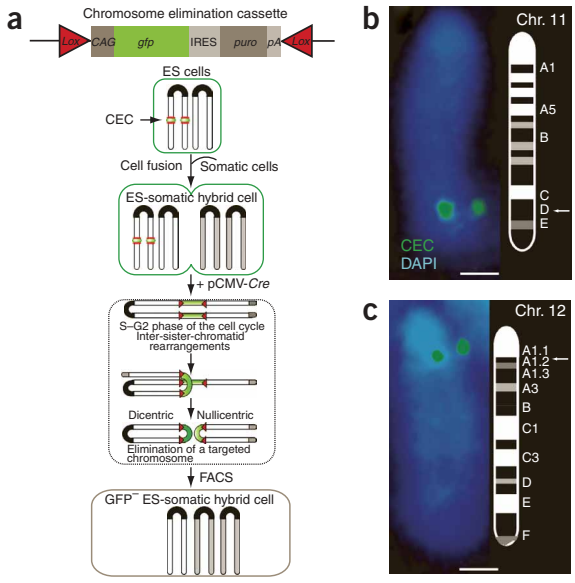

Figure 1 | ESCs carrying the CEC and cell fusion with somatic cells. (**a**) Scheme of cell fusion between ES and somatic cells, and targeted elimination of the CEC-tagged ESC chromosome. (**b**) Mapping of the CEC on chromosome 11 of ESCs. (**c**) Mapping of the CEC on chromosome 12 of ESCs. White arrow indicates CEC integration site detected by green signals. Scale bars, 1 μm.

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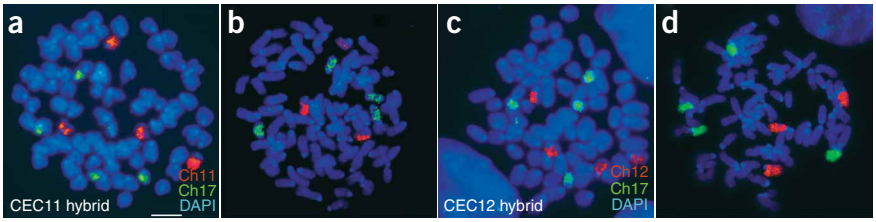

Figure 2 | Chromosome painting of metaphase nuclei after elimination of chromosome 11 or 12. (**a**) Chromosome painting of CEC11 hybrid cells before Cre treatment. Four chromosome 11s in a nucleus are recognized by red painting signals. (**b**) Chromosome painting of Cre-treated CEC11 hybrid cells. Chromosome elimination resulted in three chromosome 11s in each nucleus, as recognized by red painting signals. (**c**) Chromosome painting of CEC12 hybrid cells before Cre treatment. Four chromosome 12s in a nucleus are recognized by red painting signals. (**d**) Chromosome painting of Cre-treated CEC12 hybrid cells. Chromosome elimination led to three chromosome 12s in each nucleus, as recognized by red painting signals. Scale bar, 10 μm.

We next examined the cells for selective elimination of chromosomes 11 and 12 from the CEC11 and CEC12 hybrid cells, respectively, which was confirmed by chromosome painting analysis with specific probes. Without Cre treatment, each CEC11 hybrid cell nucleus analyzed contained four copies of chromosome 11 and four copies of chromosome 17 (**Fig. 2a**). After Cre treatment, we could detect only three copies of chrosomosome 11, whereas we detected four copies of chromosome 17 in each case (**Fig. 2b**). We observed a similar situation for chromosome 12 elimination in CEC12 hybrid cells (**Fig. 2c**,**d**). To investigate the specificity of targeted elimination and integrity of nontargeted chromosomes, we karyotyped Cre-treated CEC11 and CEC12 hybrid cells. CEC11 hybrid clones had a karyotype of 79, XXXY (–11; missing chromosome 11) (**Supplementary Fig. 2** online), whereas the CEC12 hybrid clone had a karyotype of 79, XXXY (–12) (**Supplementary Fig. 2**). Taken together, our analysis clearly demonstrates that individual autosomes can be efficiently eliminated from ES-somatic hybrid cell nuclei. Notably, chromosome elimination frequency varied between the two integration sites with 26.1% of GFP-negative CEC11 hybrid cells showing a loss of chromosome 11, and 88.4% of GFP-negative CEC12 hybrid cells exhibiting a loss of chromosome 12 (**Supplementary Table 1** online). Irrespective of this, our method has the broad potential for targeting deletion of any desired chromosome or group of chromosomes.

To begin to address (i) the efficiency of tagging a specific chromosome with the CEC by targeted homologous recombination, and (ii) the effects of eliminating both copies of the tagged ESC chromosomes from hybrid cells, we next targeted both chromosome 6, which contains the *Nanog* gene and the *Gt(ROSA)26Sor* locus (**Fig. 3a**). Using this approach we were also able to investigate whether reprogramming of the somatic genome is sufficient to confer stable stem cell properties in the absence of an ESC genome. Expression of *Nanog* is essential for maintaining a renewable pluripotent state in ESCs and embryonic cells[5,6], and it has been shown previously that silent somatic copies of *Nanog* were reactivated by nuclear transfer and cell fusion[5]. The *Gt(ROSA)26Sor* locus is a domain upstream of *Nanog* where homologous recombination is known to occur efficiently.

We electroporated a targeting vector with a CEC containing *Pgk-neo/IRES.gfp* gene subcloned into pROSA26-pA (**Fig. 3a**) into HM1 ESCs. Out of 18 G418-resistant clones tested, two were correctly targeted ($CEC6^{tg/+}$) producing a knock-in–specific 2.3-kb *Eco*RV fragment, which we detected by Southern blot analysis (**Fig. 3b**) and confirmed by FISH analysis (**Supplementary Fig. 3** online). We divided these clones and grew them for an additional 10 d under high G418 concentration. Of five clones tested, all were homozygous for CEC6 ($CEC6^{tg/tg}$) as shown by loss of the 11-kb wild type–specific band on Southern blot (**Fig. 3b**).

We next used the $CEC6^{tg/tg}$ ESCs with normal karyotype, to prepare hybrid cells with adult thymocytes that were isolated from female JF1 mice. (Experiments with mice were performed according to the institutional guidelines of Kyoto University.) After ES-somatic cell fusion, we selected GFP-positive hybrid clones on hipoxanthine, aminopterin and thymidine medium. Three days after Cre treatment, chromosome painting analysis of total hybrid cells showed that 2.2% (5/227) cells had only two copies of chromosome 6, indicating elimination of both ESC-derived CEC-tagged copies of chromosome 6 (**Fig. 3c**). Seven days after Cre treatment, we sorted GFP-negative cells by FACS and amplified them into single cell–derived clones. Notably, out of 20 clones analyzed by chromosome painting, 19 had cells with three copies of chromosome 6 in each nucleus (**Fig. 3d**). One clone retained a metacentric chromosome indicating formation of an isochromosomal Robertsonian translocation by duplication of chromosome 6 (**Fig. 3e**). The lack of hybrid clones with two copies of chromosome 6 at this stage suggests that genetic imbalance of gene dosage in tetraploid cells with only two copies of chromosome 6 might hamper cell survival and proliferation. We obtained the same result in independent triplicate experiments. The three copies of chromosome 6 were of somatic (JF1) origin, as determined by PCR analyses of 129-JF1 interspecific DNA sequence polymorphisms at proximal *D6Mit183*, central *D6Mit102* and distal *D6Mit14* (**Supplementary Fig. 3**). In contrast to chromosome 6, both somatic (JF1) and ESC (129)–derived chromosomes 1, 2 and 17 were retained, as detected by DNA polymorphism–based PCR analyses with specific primer sets, D1Mit234, D2Mit493 and D17Mit133 (**Supplementary Fig. 3**).

To confirm that both copies of the ESC-derived chromosome 6 had been eliminated, we amplified *Nanog* transcripts by reverse transcriptase–PCR (RT-PCR). An exon-specific sequence polymorphism renders ESC-derived products resistant to digestion with *Fok*I, whereas JF1 somatic cell–derived products are sensitive to this enzyme[6]. Before Cre treatment, we detected both an ESC-derived 570-bp band and the somatic cell–derived 326- and 244-bp bands, but after Cre treatment we only detected the somatic cell–derived bands (**Fig. 3f**). We obtained a similar result for the *Stella* gene, which is expressed in ESCs and primary germ cells, and also maps to chromosome 6 (ref. 7). Only somatic cell–derived *Stella* RT-PCR products could be detected from Cre-treated hybrid cell RNA producing *Hin*fI digestion fragments of 141, 136 and 33 bp (**Fig. 3f**). Thus, both ESC-derived copies of chromosome 6 were selectively eliminated from the hybrid cell nucleus. It is unclear whether in Cre-treated hybrid cells the third copy of chromosome 6 results from duplication of JF1 somatic chromosome 6.

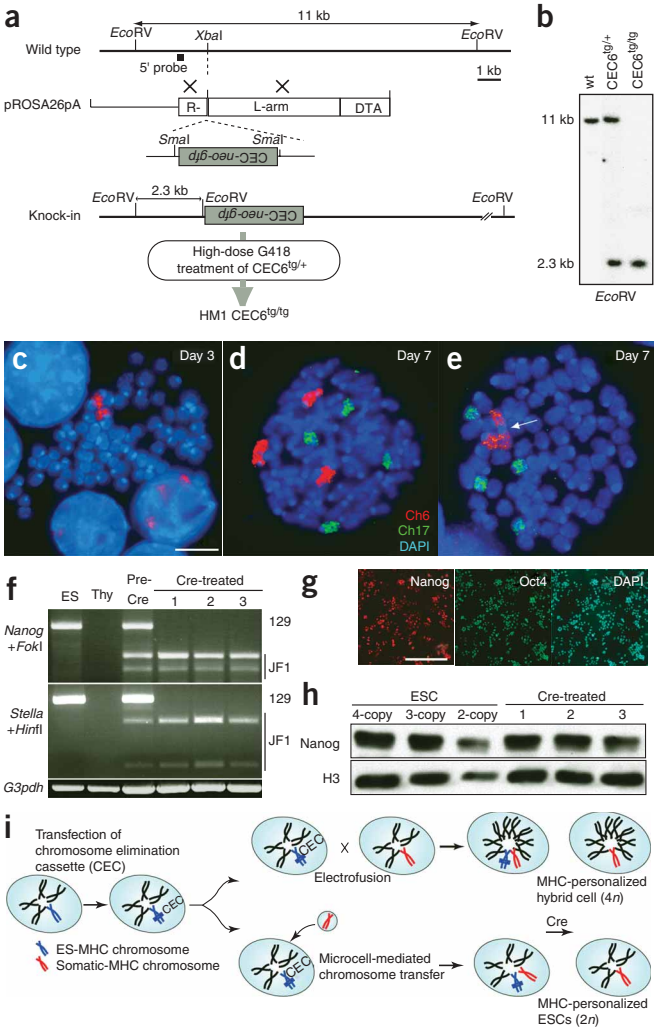

Figure 3 | Targeted elimination of a pair of ESC-derived chromosome 6s in a hybrid cell nucleus. (**a**) Scheme of integration of the CEC-*neo-gfp* cassette into the *Gt(ROSA)26Sor* locus by homologous recombination ($CEC6^{tg/+}$). ESCs homozygous for CEC ($CEC6^{tg/tg}$) were selected by high-dose G418 treatment. (**b**) Southern blot hybridization analysis of homologous recombination in the $CEC6^{tg/+}$ and $CEC6^{tg/tg}$ ESCs. The 11-kb band is specific to the wild-type allele, whereas the 2.3-kb band is specific to the knock-in allele. (**c**) Chromosome painting of $CEC6^{tg/tg}$ hybrid cell metaphase spread having two chromosome 6s (red) in a nucleus 3 d after Cre treatment. Scale bar, 10 μm. (**d**) Chromosome painting of GFP-negative $CEC6^{tg/tg}$ hybrid cells retaining three chromosome 6s (red) and four chromosome 17s (green) in a nucleus 7 d after Cre treatment. (**e**) Chromosome painting of GFP-negative $CEC^{tg/tg}$ hybrid cells carrying a bi-armed chromosome formed by Robertsonian translocation between two chromosome 6s, and a normal chromosome 6 in a nucleus 7 d after Cre treatment. (**f**) Determination of the origin of *Nanog* and *Stella* transcripts by RT-PCR analysis of polymorphic products. (**g**) Expression of Nanog and Oct4 in the hybrid cells missing ESC-derived chromosome 6s by immunocytochemical staining. Scale bar, 20 μm. (**h**) Expression level of Nanog in Cre-treated hybrid cells by western blot. (**i**) Scheme of generating personally MHC-matched stem cells.

Notably, the GFP-negative hybrid cells deficient for ESC-derived copies of chromosome 6 were capable of surviving in an undifferentiated state, verified by positive immunocytochemical staining with antibodies against Nanog and Oct4 (**Fig. 3g**). In fact, the relative Nanog expression in three independent Cre-treated hybrid clones was approximately 1.5 times that in ESCs (**Fig. 3h**). This confirmed that ESC fusion–induced epigenetic reprogramming of the somatic cell–derived *Nanog* gene is sufficient for maintaining the undifferentiated state of hybrid cells with no contribution of ESC-derived *Nanog*. Pluripotency of Cre-treated hybrid cells was shown by formation of teratomas (**Supplementary Fig. 4** online) containing multilineage tissues expressing specific marker RNA and protein (**Supplementary Fig. 4**). It is, however, unknown whether the hybrid cells can contribute to generating chimeric mice.

In regenerative medicine, personally major histocompatibility complex (MHC)-matched stem cells are eagerly awaited as a source for producing replacement tissues with reduced likelihood of immunological rejection. The majority of MHC class I and class II genes are clustered on human chromosome 6 and mouse chromosome 17. Selective elimination of ESC-derived MHC gene–containing chromosomes from ES-somatic hybrid cells may provide a source of personally MHC-matched hybrid cells (**Fig. 3i**). The CEC approach can be developed further to directly generate MHC-personalized diploid ESCs by targeted elimination of ESC chromosomes harboring the MHC genes, and replacing both copies with somatic cell–derived MHC chromosomes using the microcell-mediated chromosome transfer technique[8] (**Fig. 3i**). Our targeted elimination of chromosomes is applicable to a variety of biomedical purposes. Generating human stem cells by introducing somatic cell–derived chromosomes with specific mutations from patients should help evaluate the causes of human diseases as well as discover appropriate drugs through pharmaceutical evaluation *in vitro*.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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