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Chromosome Engineering in Mouse Embryonic Stem Cells: Addition and Elimination of Targeted Chromosomes

Masako Tada
Division of Chromosome Engineering & Therapeutics, Chromosome Research Center, Tottori University, Nishi-cho 86, Yonago, Tottori 683-8503 Japan

1. Introduction

Human pluripotential stem cells, including both embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells), possess self-renewing potency or the ability to differentiate into virtually any type of somatic cell. These features make them particularly advantageous as sources from which to generate specific types of human tissue cells *in vitro* for use in drug development and regenerative medicine. In most cases, however, human pluripotential cell lines and especially human ES cells can only be used in cell-based applications because of ethical issues. Animal models are therefore sought as an alternative to using human cells. An increasingly popular non-human primate model is the common marmoset (*Callithrix jacchus*). The recent successful creation of lentivirus-mediated transgenic marmosets provides a new animal model for human disease offering the powerful advantage of a close genetic relationship with humans (Sasaki et al., 2009), though this technique is not yet sufficiently developed for common use, and the numbers of monkeys available for experiments are limited. Mouse ES cells, meanwhile, still have great value as a research tool, even after the development of human pluripotent cells, as they can be used to create chimeric mice, achieve germline transmission, and generate normal offspring.

The available genetic engineering technologies often employ an embryonic manipulation approach in mice, using mouse ES cells to examine the gain-of-function or loss-of-function effects associated with certain chromosomal regions *in vivo*. Recently, we have developed chromosome elimination cassettes (CEC) using a Cre-inverted loxP system that was first used in mouse ES cells. In this system, transient *cre* expression can initiate immediate chromosomal loss over the course of a few cell cycles in the recombinant cells. This technology was developed to clarify chromosomal function through observing loss-of-function at the chromosomal level. In mammalian cells, chromosome composition and gene dosage are kept stable because large chromosome-wide deletions are usually fatal. Accordingly, we first applied the Cre-inverted loxP system to tetraploid cells composed of mouse ES cells fused with mouse somatic cells to generate conditions conducive to large-scale chromosomal imbalance. The CEC-tagged chromosomes could be targets of Cre-dependent chromosome elimination. In addition, we have demonstrated that the Cre-inverted loxP system enhances cohesion between a loxP site and an adjacent inverted loxP,

which induces a remarkable degree of spontaneous mitotic recombination around loxP sites. These characteristics may also help us to resolve the mechanisms involved in spontaneous mitotic recombination events leading to chromosomal deletion during tumorigenesis. This review discusses the development of the chromosome elimination method over the last few years and provides examples of the emerging practical use of mouse ES cell lines containing CEC-tagged chromosomes as a chromosome engineering technology.

Recent advances in the opposite direction, that is, toward the insertion of chromosomes into cells as a chromosome engineering technology, are also discussed here. Human artificial chromosome (HAC) vectors have been used to transport intact foreign chromosomal segments. The Cre-loxP system is frequently employed in this form of chromosome transfer technology as well. HAC contains an acceptor loxP that promotes insertion of the chromosomal region through chromosome translocation with the donor loxP-tagged chromosome (Smith et al., 1995). If undesired chromosomal regions exist at the ends of the loxP-tagged donor chromosome, the regions can be trimmed using telomere-directed truncation technology; for the efficient application of this technique, chicken DT40 cells are often used as high homologous recombination-proficient cells. At the end of this process, the desired region is introduced into the final host cells (Dieken et al., 1996; Kuroiwa et al., 1998). In any step of this process toward the production of the final transformants, an HAC can be sequentially transferred from one type of cell to the other by means of the microcell-mediated chromosome transfer (MMCT) technique (Kuroiwa et al., 2000). MMCT requires a high level of skill, but can be used to produce 1:1 cell fusion products between micro-cells, which contain a recombinant chromosome, and host cells at high frequencies. Though some trials have been started, this remains a significant problem in chromosome transfer technology (Katoh et al., 2010). As a consequence, trans-chromosomal mice generated by germline transmission of an HAC have been created *via* the formation of chimeric mice with mouse ES cells that stably possess a defined chromosomal region on an HAC (Kuroiwa et al., 2002). Using this chromosome transfer technology with pluripotential cells offers advantages supporting the identification of the functions of given chromosome segments on the basis of gain-of-function and the repair of impaired genome-wide functions in the host cells. By observing the functional differences between wild-type cells and transformants, researchers can identify the responses to given stimuli and address these effects via chromosome transfer. These cells are not expected to over express any particular molecule on the transferred chromosome and are thought to exhibit more-or-less physiological responses to transcriptional regulation *in-vivo*. Accordingly, chromosome-transferred cells might sometimes constitute a more useful assay system than single-gene recombinant cells that highly express a single target molecule. Moreover, parallel inheritance of a human chromosome by cells from another animal species after elimination of the parallel chromosomal region makes it possible to create humanized animals.

The physiological relevance of biological analyses using CEC or HAC technology is actually demonstrated by the epigenetic reprogramming activity that occurs in host pluripotent cells. Reprogramming factors operating in pluripotent cells can impose epigenetic modifications on foreign somatic chromosomes that have been introduced by cell fusion, making their cells equivalent to host pluripotent cells. Without this activity, introduced chromosome segments would never be able to behave like their homologues or orthologues in the given cells *in vitro* and *in vivo*. Our previous important findings on the epigenetic reprogramming potencies working in mouse ES cells are discussed first.

2. Epigenetic reprogramming potencies in mouse ES cells

In keeping with the classic epigenetic landscape model, it was long believed that epigenetic marks were placed into the genome step by step during a developmental program and that the epigenetics of somatic nuclei would never revert to a pluripotential, multipotential, or bipotential state. The successful creation of adult normal frogs by nuclear transfer from highly specialized tadpole intestinal cells into ultraviolet-light-irradiated oocytes clearly demonstrated that developmental programs can be completely reversed (Gurdon, 1962). The first successfully cloned mammal was made by fusing a G0 cell with an enucleated unfertilized oocyte (Wilmut et al., 1997). As technology improved, it became possible to replace a mouse oocyte nucleus with a mouse somatic nucleus through enucleation; this led to the successful creation of cloned mice (Wakayama et al., 1998). This process is called somatic-cell nuclear transfer, and is now widely used. Complete epigenetic reprogramming *in vivo* through nuclear transfer creates another means by which the specific genotype of an animal can be propagated in the absence of mating.

Animal cloning suggested that epigenetic restoration was occurring in the somatic nuclei during embryonic development, but the these epigenetic changes were first analytically visualized through cell fusion between somatic cells and undifferentiated cells. Every female mammalian somatic cell possesses an inactivated X chromosome as a gene dosage compensation mechanism between XX female cells and XY male cells. To maintain X chromosome inactivation, numerous molecules, including the non-coding RNA *Xist*, are accumulated on the inactive X chromosome *in cis*. Moreover, DNA cytosine methylation of the *Xist* gene is kept at a low level on the inactivated somatic X chromosome. The somatic X chromosome is converted from an inactive state to an active state through cell fusion with mouse embryonic carcinoma cells (EC cells), in which case the fused cells acquire undifferentiated-cell morphology (Takagi et al., 1983).

As a next step, we demonstrated that *de novo* DNA cytosine methylation of the *Xist* gene might silence the *Xist* allele on the previously inactivated somatic X chromosome and initiate conversion of the X chromosome from an inactive state to an active state in somatic hybrid cells between certain kinds of EC cells and female somatic cells (Mise et al., 1996). By that time, however, we were asking whether the mouse EC cells had lost an important factor required for the maintenance of the X inactivation state through carcinogenesis or possessed dominant factors that converted the somatic nucleus to a host EC cell-like nucleus. To resolve this question, we designed a cell fusion experiment involving mouse embryonic germ cells (EG cells) and mouse somatic cells (Tada et al., 1997). The mouse EG cells, derived from primordial germ cells from embryonic-day (e)11.5 to e12.5 female embryos, had lost the epigenetic differences, also known as genomic imprinting, between the homologous chromosomes derived from the two parents. Inactivation marks on the X-chromosome and genome-wide CpG methylation had already been eliminated from the mouse gonadal EG cells. These cells could contribute to mouse development, but the resulting chimeric embryos were abnormal because of the deletion of the previous genomic imprints (Tada et al., 1998). Thus, unlike mouse EC cells, mouse EG cells are not derived through carcinogenesis, and they maintain a set of the epigenetic properties of the original cells. Several important characteristics of the epigenetic reprogramming activities working in embryonic pluripotent cells were first analytically demonstrated at the molecular level through cell fusion experiments using gonadal mouse EG cells and mouse somatic cells. The mouse EG cells contribute pluripotency to the resulting somatic nuclei, which in turn grants

them several other EG cell-like characteristics, including the following: (1) reactivation of inactivated X chromosome derived from female somatic cells, (2) competence for embryonic development, (3) deletion of genome-wide DNA cytosine methylation, (4) deletion of methylation marks from several imprinted and non-imprinted genes, and (5) demonstrable reactivation of the repressed paternally imprinted allele of *Peg1/Mest*.

We then showed that somatic cells can acquire a pluripotent state after being fused with mouse ES cells (Tada et al., 2001). Using thymocytes from female mice that contained a GFP reporter transgene driven by the promoter of mouse *Oct4,* we monitored *Oct4* reactivation by an *Oct4–gfp* reporter. In contrast to germ cells, mouse ES cells do not possess demethylation activity for imprinted genes in fused tetraploid cells, as far as we could determine. Interestingly, methylation imprints on mouse ES cell chromosomes were erased in the hybrid cells made from mouse gonadal EG cells. This evidence shows that mouse EG cells possess dominant factors leading to the erasure of methylation imprints. The latter fusion experiments included our first use of intersubspecies hybrid cells, made from *Mus musculus domesticus* ES cells and *Mus musculus molossinus* thymocytes (Tada et al., 2003; Kimura et al., 2004; Kimura et al., 2002; Hatano et al., 2005). Frequent DNA sequence polymorphisms between these two subspecies allowed us to monitor the origin of the RNA and DNA derived from the somatic nuclei of the tetraploid hybrid cells. Using this mouse ES cell-somatic cell fusion system, we demonstrated (6) reactivation of some pluripotency-associated genes derived from somatic genomes (*i.e.*, *Oct4*, *Nanog*, and *Tsix*) and (7) conversion of histone modification of somatic cell-derived chromatin to a pluripotent state. The reprogrammed somatic genomes in the hybrid cells made from mouse ES cells became hyperacetylated at histones H4 and H3 and globally dimethylated and trimethylated, with respect to the lysine residue K4, at H3. Such epigenetics are known as typical modifications for transcriptionally active regions. Later, another group demonstrated that overexpression of *Nanog* substantially enhanced fusion-based nuclear reprogramming (Silva et al., 2006). *Nanog* is known to be an important transcriptional factor involved in maintaining pluripotency (Chambers et al., 2003; Mitsui et al., 2003). We have previously shown that *Nanog* expression is controlled by *Oct4* and *Sox2* (Kuroda et al., 2006), which regulate the pluripotency of mouse ES cells in a dose-dependent manner (Hatano et al., 2005). Thus, we expected that two copies of endogenous *Nanog* mRNA might not be sufficient to make the tetraploid cells pluripotent during the initial full cell cycles after cell fusion with somatic cells when extrinsic *Nanog* was fully reprogrammed. We addressed this question by attempting the complete elimination of two copies of mouse ES cell-derived *Nanog*-bearing chromosome 6s in ES cell-somatic cell hybrid cells (Matsumura et al., 2007). The results clearly demonstrated that *Nanog* expression from reprogrammed somatic genomes could efficiently maintain pluripotency in mouse ES cell-somatic cell hybrid cells, but that at least three out of four copies of chromosome 6s were required to keep tetraploid cells pluripotent. This will be discussed in greater detail below.

ES cell-specific epigenetic profiles are predominantly regulated by intrinsic factors, so that the host cell's epigenotypes can be exposed to foreign DNAs and histones in ES cell-somatic cell hybrids. Several reprogramming core factors have already been identified, including *Oct4*, *Sox2*, *c-Myc*, and *Klf4* (OSCK). Overexpression of OSCK or OSK via virus- or nonvirus-mediated gene transformation can reprogram mouse and human somatic cells into pluripotential cells; cells reprogrammed through this means are known as iPS cells (Nakagawa et al., 2008; Takahashi et al., 2007a; Takahashi et al., 2007b; Takahashi & Yamanaka, 2006). The use of this *in-vitro* somatic reprogramming technology to produce

human iPS cells is expected to be the long-awaited breakthrough that will allow researchers to produce syngenic tissue cells from personalized stem cells obtained from patients who require tissue-cell transplants. In the clinical setting, the propensity of iPS cells to form tumors is the main problem remaining to be solved, but functional somatic cells derived from human iPS cells could contribute to *in-vitro* analysis. Human iPS cells are currently regarded as a promising tool, satisfying the pharmacological industry's need for scalability and physiological relevance. Many researchers are planning to acquire patient-specific human iPS cell lines from somatic cells carrying genetic dysfunctions in order to develop medical compounds or gene therapies that reduce the symptoms associated with these genetic dysfunctions. Primary cultured somatic cells derived from patients are now becoming available from cell banks, helping us to produce models of gene therapy or chromosome-mediated therapy based on human iPS cells. Some kinds of biological analysis require accurate assessment of the phenotypic and physiological differences between normal cells and genetically affected cells in an identical genetic background. Even if normal cells were available from a patient with a genetic dysfunction, it might be difficult to compare the iPS cell line established from the affected tissue with that established from neighboring unaffected tissue because of the wide clonal variation among the iPS clones established through factor-mediated reprogramming. Thus genetically manipulated pluripotential cells created through knock-out, knock-in, transgene, or another molecular engineering technology might still provide some advantage over iPS cells because normal controls for the manipulated cells are always available.

3. General chromosome manipulation tools

3.1 Cre-loxP system

The Cre-loxP system is particularly useful for creating new chromosomes carrying site-specific deletions, duplications, inversions and translocations. A single loxP site contains two 13-bp inverted repeats (5′-ATAACTTCGTATA-3′ and 5′-TATACGAAGTTAT-3′) flanking an asymmetric 8-bp core sequence (5′-GCATACAT-3′). The central core sequence defines the orientation of the loxP site. Thus the core sequence in inverted-loxP can be described as 5′-ATGTATGC-3′. One Cre (causes recombination) recombinase monomer binds each inverted repeat. The synaptic complex is first formed through dimerization of the loxP-bound Cre molecules, and then Cre catalyzes DNA strand exchange between the homologous core regions *via* a Holliday intermediate. Only when two sites are placed as direct repeats on the same DNA strand does Cre induce restricted deletion of the DNA placed between the two loxP sites. Using this system, the targeting of a specific undesired DNA sequence can be Cre-dependently induced in a specific tissue type or cell type both *in vivo* and *in vitro* (Branda & Dymecki, 2004; Mills & Bradley, 2001). The Cre-loxP system also enables the induction of chromosome-wide recombination *in cis*, leading to megabase deletion, and can induce interchromosomal exchange between any loxP-tagged chromosomes, resulting in targeted chromosomal reciprocal translocation. Such *in-trans* recombination is induced to allow transfected exogenous sequences to integrate into the host genome through mitotic recombination at loxP sites (Figure 1A).

In addition from the experimental machinery, a native cellular mechanism for mitotic recombination exists in mammalian cells. For spontaneous mitotic recombination to occur, two DNA strands possessing high similarity first align spontaneously in a nucleus and then are exchanged through the repair of spontaneous DNA double-strand breaks between them.

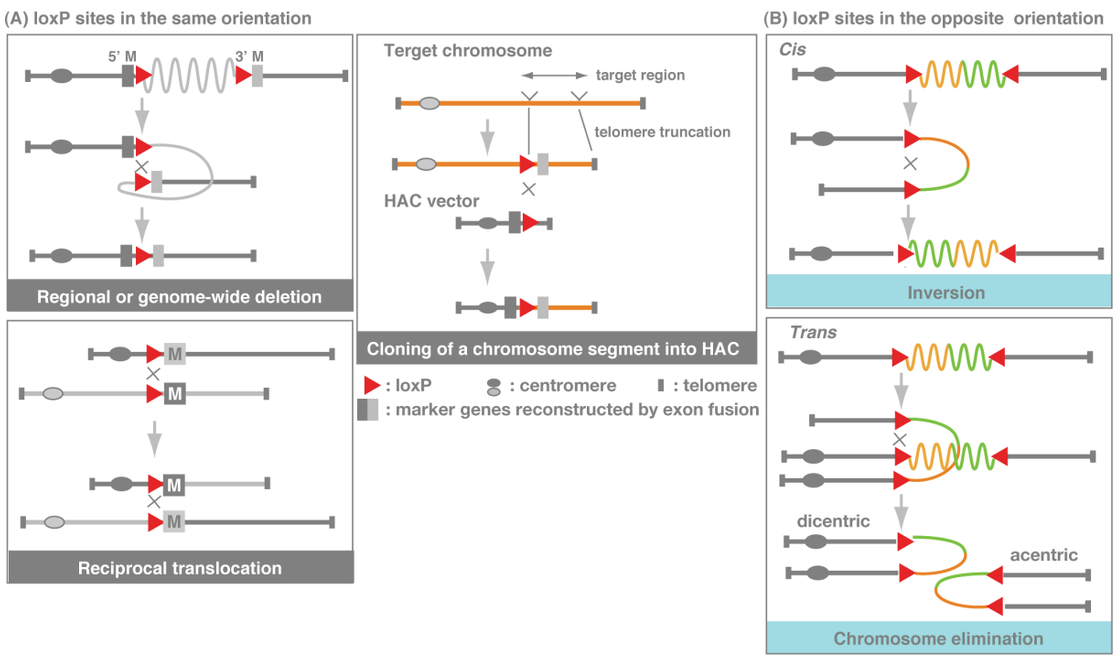

Fig. 1. Chromosome engineering technologies by means of Cre-loxP system. (A) Targeted insertion of loxP sites is practically used to induce a chromosome-wide deletion or a reciprocal translocation. Using sequential gene targeting, two loxP sites are introduced into two regions on the same or different chromosomes, resulting in chromosome-wide deletion or chromosome translocation, respectively. A loxP are often combined with 5' portion of a marker gene, while the other one are combined with the remaining portion of the marker gene. The marker genes reconstructed by exon fusion permit cell growth of the Cre recombinants. The Cre-loxP system is also applied to induce chromosome translocation between a desired chromosomal segment and an HAC vector. (B) Cre-inverted loxP system is used to induce elimination of the targeted chromosomes in the living cells. This system often induces inversion of the DNA region that has been placed between two loxP sites.

When two different chromosomes are targets of mitotic recombination, the daughter cell received a new recombinant created through balanced reciprocal translocation. In other cases, mitotic recombination causes the production of daughter cells carrying uniparental disomy. Even if recombinant cells do not contain any genetic imbalance, uniparental disomy sometimes causes detrimental effects because of the genomic imprinting phenomenon in mammals. Genomic imprinting, also known as epigenetic marking, leads to exclusive uniparental expression of the imprinted genes. Thus, uniparental disomy of imprinted regions causes either overexpression or nonexpression of the imprinted genes.

3.2 Cre-inverted loxP system

In the Cre-inverted loxP system, on the other hand, where the core sequence of the second loxP site is inverted, the DNA array placed between two loxP sites is often inverted through parallel synapses and recombination at the loxP sites located on the same DNA strand (Spitz et al., 2005). This inversion event yields intact recombined loxP sites, which will become targets of the second recombination. Moreover, Cre can initiate recombination between the

loxP sites located on a pair of sister chromatids, resulting in derivation of a dicentric chromosome and an acentric chromosome. The subsequent cell divisions yield an accumulation of cells exhibiting targeted chromosomal loss (Lewandoski & Martin, 1997) (Figure 1B). We have developed CEC plasmid vectors containing a pair of loxP sites in an inverted orientation, which reproducibly induced hemizygous or homozyous loss of CEC-tagged chromosomes (Matsumura et al., 2007; Otsuji et al., 2008). To isolate transformants of CEC, a ubiquitously expressed drug-resistant gene and a green fluorescent protein (GFP) reporter gene were inserted in the into the Cre-inverted loxP sequence.

4. Chromosome elimination in mouse pluripotential cells

In mammalian cells, chromosome composition and gene dosage are kept stable, and large-scale chromosome-wide deletions are usually fatal. To create a non-fatal large-scale chromosomal imbalance, we applied the Cre-inverted-loxP system to tetraploid hybrid cells derived from mouse ES cells and somatic cells. As a first step, we prepared multiple stable CEC transformants of mouse ES cells, in which CEC-tagged chromosomes become targets of selective elimination. In the Cre-inverted loxP system, where the core sequence of the second loxP site is inverted, the inverted loxP sites are able to form parallel synapses between sister-chromatids after DNA synthesis, which produce a dicentric chromosome and an acentric chromosome as Cre-induced recombination products (CRPs). The CRPs are visible in many metaphases one to three days after Cre treatment. As a consequence, CRPs are eliminated in the course of the cell divisions that follow (Figure 1B). Such targeted chromosomal loss has been induced in progeny carrying a Y-transgene and containing an accidentally inverted loxP within a multi-copy array of directly repeated loxP sites (Lewandoski & Martin, 1997). Thus conditional elimination of targeted chromosomes might be technically inducible in mice, though viable offspring cannot be expected if any one of the intact autosomal chromosomes is eliminated.

4.1 Chromosome elimination cassette (CEC)

The CEC contains a ubiquitously expressed GFP-encoding gene and a drug-resistant gene between a loxP site and an inverted loxP site. The reporter genes were intended to allow researchers to isolate CEC-transformants as GFP-positive and drug-resistant cells, while Cre recombinants missing CEC-tagged chromosomes can be selectively obtained as GFP-negative cells through fluorescence activated cell sorting (FACS). Each sorted cell can be clonally expanded as an independent Cre-recombination product for further analysis.

4.2 Methods

4.2.1 CEC-tagging

Two kinds of CECs and their transgenic ES cell lines have been created: pCEC-CAG-*gfp*/IRES.puro-pA (CECpuro) and pCEC-Pgk-*neo*/IRES.*gfp*-pA (CECneo). The linealized DNAs of CECpuro and CECneo were electroporated into mouse ES cells to allow for the isolation of transformants through purimycin and G418, respectively. The transformants of linealized CEC vector DNA were isolated from HM1 mouse ES cells (129/Ola: *Mus musculus domesticus*) deficient in the *Hprt* gene. In such cases, CEC can be introduced into the desired part of the chromosome by homologous recombination. Thus fused cells created from mouse ES cells and wild-type somatic cells were isolated by means of HAT selection medium. All of the stable transformants were analyzed for their CEC-integration sites using

the fluorescence-*in-situ*-hybridization (FISH) method. The location of FISH signals was determined on the basis of the G-banded pattern of the chromosomes. By now, CEC integration sites have been observed on 13 chromosomes out of the 19 pairs of autosomes and sex chromosomes of laboratory mice. These mapping data and chromosome identifications have been reported previously (Tada et al., 2009). The CEC-tagged chromosomes could be future targets for creating monosomy or uniparental disomy in a Cre-dependent manner.

4.2.2 Whole cell fusion

ES cell-like hybrid cells derived from mouse ES cells and mouse somatic cells are obtained by means of electric fusion followed by HAT selection. The electric fusion protocol has been described previously (Tada & Tada, 2006a, b). Only *Hprt*-positive cells can grow in HAT selection medium, so its use results in selective growth of the fused cells derived from *Hprt*-negative ES cells and wild-type somatic cells. Hybrid cells derived from CECneo-transgenic ES cells and CECpuro-transgenic ES cells can be isolated through co-treatment with G418 and puromycine after electric cell fusion. JF1 mice possessing a *Mus musculus molossinus* genetic background were often used as somatic cell donors so that the origin of the chromosomes derived from somatic cells in the tetraploid cells could be determined.

4.2.3 Cre treatment

The Cre expression vector pCMV-*cre* (Gibco-BRL, Invitrogen, Carlsbad, CA, USA) was modified into pCAG-*cre* and then transfected into CEC-tagged mouse ES cell hybrid cells by means of Lipofectamine™ 2000 (Invitrogen) or Nucleofector® (Amaxa, Basel, Switzerland). The best program and DNA content for nucleofection were A13 and 5 mg of DNA to $2x10^6$ cells. A high transfection rate was obtained: more than 60% of surviving cells showed transgene expression within one day after nucleofection. Consistent with this data, efficient induction of CRPs of CEC chromosomes was recorded one day after nucleofection. Further, multi-day culture yielded an accumulation of cells missing CEC-tagged chromosomes.

4.3 Whole chromosome elimination

First, we attempted single chromosome elimination in CECpuro transformants of mouse ES cell hybrid cells with mouse somatic cells (Matsumura et al., 2007). After conventional lipofection of the Cre expression vector, GFP-negative cells were isolated by FACS. All clones proliferated from a single GFP-negative cell were used for FISH analysis. The results clearly showed that CEC-tagged chromosomes were selectively eliminated in a Cre-dependent manner. Next we attempted to eliminate two chromosomes at once (Otsuji et al., 2008). We generated four sets of fusion clones derived from CECpuro-tagged mouse ES cells and CECneo-tagged mouse ES cells through cell selection with G418 and puromycin. In the case of the fused mES cells containing both CECpuro-tagged chromosome 12 and CECneo-tagged chromosome 17 (CEC12/17), flowcytometric analysis after conventional lipofection of the Cre expression vector showed only a 5% increase in GFP-negative cells in 7-day-old cultures. After nucleofection of the Cre expression vector, in contrast, nearly 50% of Cre-treated cells were GFP-negative hybrid cells. FACS-mediated isolation of GFP-negative cells was successively achieved by recovering the 78,XXYY, -12, -17 cell clones as Cre-recombinants with 96% purity from 80,XXYY tetraploid cells containing four chromosome 12s and four chromosome 17s. Similarly, we detected the loss of one set of chromosomes

each from CEC11/17, CEC6/11 and CEC6/12 through FISH chromosome painting. In these hybrid cells, asynchronous chromosomal loss was also observed, even though two CEC regions were exposed to Cre activity in the same nucleus. This asynchrony gradually decreased over 5 days, however. In any case, following the DNA replication stages will provide Cre enzymes with access to loxP sites. As a next step, we need to precisely identify the kinds of autosomes to which chromosome elimination technology can be applied.

4.4 Homozygotic chromosome elimination

CEC6 mouse ES cells were created by means of the homologous recombination technology known as knock-in, through which CECneo vector was inserted into the *Rosa26* locus on mouse chromosome 6 (Matsumura et al., 2007). We prepared mouse ES cells that were homozyous for a CEC-tagged region through either mitotic recombination between homologous chromosomes or a combination of loss of wild-type chromosome and duplication of CEC-tagged chromosomes. Because there are two expressed copies of *neo* in a CECneo transformant, the homozygotes created from this transformant can be grown in the presence of high doses of G418. Using this system, we tried to demonstrate that the reprogrammed chromosome 6s derived from JF1 somatic cells could maintain an undifferentiated state without mouse ES cell-derived chromosome 6s as mentioned above. Cre recombinants missing both copies of chromosome 6s derived from mouse ES cells only expressed *Nanog* mRNA from JF1-derived chromosome 6s, and maintained their pluripotency. This fact clearly demonstrates that reprogrammed somatic chromosomes can functionally replace the ES chromosomes in hybrid cells derived from mouse ES cells and somatic cells. For tetraploid cells to survive and maintain their self-renewing potency, however, three copies of chromosome 6s were likely to be required. We found that every Cre-transformant possessed three chromosome 6s due to spontaneous duplication of somatic chromosome 6. Thus epigenetic reprogramming of the somatic cell–derived *Nanog* gene is sufficient, but the group of genes encoding chromosome 6 including *Nanog* might be sensitive to gene-dosage regulation.

4.5 Partial chromosome deletion

Theoretically, in the Cre-inverted loxP system, partial chromosomal deletions are not expected. Nevertheless, more than 30% of CEC6 mouse ES cells were observed to have turned into GFP-negative cells in 5-day-old Cre recombinants due to partial deletion, whereas whole chromosomal loss occurred at low frequencies (around 5%). It is therefore evident that the frequencies of regional mitotic recombination between short identical sequences in the genomic region of chromosome 6s are enhanced *via* a scaffold formation through Cre-mediated antiparallel cohesion between loxP sites (Otsuji et al., 2008). Thus a small deletion is less likely to be fatal; in fact, some diploid Cre recombinants isolated from CEC6 mouse ES cells maintained their self-renewing potencies and expressed both *Nanog* and *Oct4*. The *Rosa26* locus occurs close to the mouse von Hippel-Lindau tumor suppressor gene *Vh1h*, an orthologue of human *VHL*, which is located on human chromosome 3p25.5. Null mutation coupled with germline mutation and chromosomal deletion of the *VHL* gene have been reported in familial VHL syndrome, which predisposes affected persons to malignant or benign tumors (Maher & Kaelin, 1997; McGrath et al., 1992; Latif et al., 1993). Based on these cytogenetic reports, VHL syndrome is also known as 3p- syndrome (Sherr, 2004). In mice, heterozygotic loss of *Vhl1* is sufficient to predispose affected mice to vascular

tumors, while homozygous loss causes embryonic lethality (Gnarra et al., 1997; Haase et al., 2001). Therefore CEC-tagged mouse ES cell clones may provide materials with which to create a loss of heterozygosity (LOH) model *in vitro* or *in vivo* by a combination of genetic mutation and inducible chromosomal deletion. We have already isolated several CEC-tagged mouse ES cell clones in which CEC-tagged regions are located near tumor suppressor genes, including *BRACA1* or *TP57* in CEC11Dpuro, *BRACA2* in CEC5puro, *P16* and *TP73* in CEC4neo, *TP53* in CEC7neo, and *VHL* in CEC6neo (Tada et al., 2009). In large chromosomal imbalances, the regions of insertion and deletion disrupt the complex interactions of many genes, not only within the chromosomal domains but also in other regions (Stallings, 2007). Thus chromosome-wide deletion technology might provide more information than simple gene disruption does as a model of deletion-mediated diseases in humans.

4.6 Future research

It has previously been shown that the frequency of mitotic recombination is about 100 times lower in mouse ES cells than it is in adult somatic cells or in isogenic mouse embryonic fibroblasts (Cervantes et al., 2002). Homozygous loss also takes place due to uniparental disomy induced either by sequential events of mitotic recombination and X segregation or by loss of functional chromosomes and duplication of the affected chromosomes. In LOH events in somatic cells, mitotic recombination predominates over uniparental disomy initiated by loss-and-duplication, for which a high level of nucleotide sequence homology is required. Despite the importance of chromosomal recombination in the LOH diseases, little is known about the properties of the junctions involved in the chromosomal rearrangements or about the responsible enzymes, because of the difficulty of inducing chromosomal deletion *in vitro* and *in vivo*. In this Cre-inverted loxP system, however, Cre significantly enhanced local rates of recombination at CEC-tagged regions. This may help to resolve the correlation between intra-chromosomal deletion events and either the flexibility of chromatin or the accumulation of junction sequences responsible for mitotic recombination. The application of CEC technology to diploid cells could help to isolate recombinants as GFP-negative clones passing through mitotic recombination more frequently rather than undergoing spontaneous recombination. This might promote better understanding of the modulator sequences responsible for the tangle structure formation and its solution mechanism that induces mitotic recombination leading to chromosomal deletions. It is clear that Cre-mediated cohesion enhances the rate of intra-chromosomal recombination in this region, but Cre is probably not involved in DNA strand exchange. Identifying the cellular factors that catalyze these recombination events will help resolve the mechanisms underlying the progression of cancers through chromosomal rearrangements.

5. Chromosome transfer into pluripotential cells

In early gene manipulation techniques, mRNA coding regions were introduced and ubiquitously expressed in a given set of cells. As a next step, endogenous transcriptional regulatory elements were connected to the open reading frames of the genes and used for developmental or tissue-specific expression. Now, to mimic the physiological expression profile of a certain gene, 100 kilobasepairs of genomic DNA are introduced into mammalian cells using bacterial artificial chromosome (BAC), P1-bacteriophage artificial chromosome (PAC), or Yeast artificial chromosome (YAC). Recently, there has arisen a need to realize the

development of larger-capacity carriers for genes or gene clusters spanning a genomic region as large as a megabase. In one example, a chromosomal region comprises a major part of the gene function shared by many genes classified as a gene family. In another example, splicing forms are regulated tissue-specifically, so that a single form may be functionally divergent when expressed in different tissues. In immunoglobin genes, moreover, VDJ segments are recombined from a vast variety of the choices aligned in tandem over a wide chromosomal region. To induce VDJ recombination against a specific immunogen under physiological conditions, this whole chromosomal region should be introduced to the host cells to make them suitable for further use. Thus the development of a chromosome-wide gene transfer system would bring researchers a new genetic tool to analyze a proper function as seen *in vivo*. Human chromosome segments have been introduced not only into human host cells but also into mouse, avian, and other types of host cells. It is evident that human chromosome segments function in the trans-chromosome mice that are created via trans-chromosome mouse ES cells. There have recently been preliminary reports claiming the creation of transchromosomic animals producing a diverse repertoire of human immunoglobulin (Kuroiwa et al., 2002; Tomizuka et al., 2000; Tomizuka et al., 1997). Theoretically, depending on the size and region of the chromosome segment in question, an extra chromosome can contribute to half of the progeny through meiosis.

5.1 Chromosome manipulation

Centromeres, together with telomeres, are essential for segregation during cell division in any eukaryotic chromosome. Telomere function is also required in each of the ends to ensure chromosomal stability. Thus, chromosomal vectors have been created as "mini-chromosomes", each containing a short chromosome segment containing a loxP site, a human chromosome-derived centromere, and two telomere ends enclosing them. A telomere consists of an array of short tandem repeats, $(TTAGGG)_n$ in humans, which form a closed loop and protect chromosome ends. Through targeted insertion of telomere repeats, a new chromosome end can be created at a desired position in the chromosome. Native centromeres from human chromosomes that are involved in vectors are likely to behave as endogenous chromosomes. Vectors containing such chromosomes are called human artificial chromosome (HAC) vectors.

5.2 Human artificial chromosome (HAC) vector

A loxP site has been introduced into the HAC to allow for Cre-mediated site-specific insertion of circular DNAs at the beginning of the HAC (Dieken et al., 1996; Kuroiwa et al., 2000). Transgenes can therefore be introduced into these loxP sites by Cre-mediated lateral recombination with a loxP site located on a plasmid-, BAC-, or PAC-vector. The acceptor loxP site on the HAC can later be used for various purposes, such as a platform for megabase-level chromosomal segments. To insert the desired chromosomal segment into the HAC, one end of each defined chromosomal region is truncated by telomere insertion, while the other end is tagged by a donor loxP site. The loxP-tagged intact or truncated chromosome is introduced into the HAC-containing host cells. Transient Cre expression is then able to combine an HAC and a donor chromosomal segment at a loxP site, leading to the creation of an HAC containing an extra-chromosomal segment. Recombinants can be selectively grown through culturing with drugs to create a complete drug-resistant gene by exon fusion technology. HACs can be introduced into mouse ES cells through MMCT

(Fournier & Ruddle, 1977; Koi et al., 1989) as a cell fusion product (Figure 1A). For further *in-vitro* use, HAC transformants could be created using human ES cells and iPS cells.

5.3 Microcell-mediated chromosome transfer

First, to manipulate the donor chromosomes, normal mouse or human cells are fused with DT40 cells, on which targeting-mediated genetic manipulation is performed. Next, the manipulated donor chromosome is transferred from the DT40 hybrids to the HAC-containing CHO cells by MMCT technology (Kuroiwa et al., 2000). Usually, HAC is maintained in the CHO cell line, which is a more capable microcell donor. When the HAC uses a centromere of human chromosome 21, the HAC vector is originally constructed in the DT40 hybrid cells, in which intact human chromosome 21 is introduced from human fibroblasts through cell fusion (Katoh et al., 2004). The HAC vector recombined through Cre treatment is maintained in CHO cells. Again, the constructed HAC vectors containing donor chromosomal segments are transferred from CHO cells to other recipient cells (Kugoh et al., 1990) such as mouse A9 cells, mouse ES cells, or the other multipotent or pluripotent cells through MMCT using standard procedures.

5.4 Trisomy 21

Trisomy 21, the most common live-born human aneuploidy, causes Down's syndrome (DS), which encompasses many clinical phenotypes including a reduced incidence of solid tumors. An extra copy of human chromosome 21 has been introduced into mouse ES cells through MMCT (Shinohara et al., 2001). The authors showed that the progeny of these chimeric mice were able to contain human chromosome 21-containing cells after small-scale deletion. Chimeric mice showed various degrees of mosaicism as regards the retention of human chromosome 21, and there was a high correlation between the retention rate of human chromosome 21 in the brain and impairment in learning or emotional behaviors. Hypoplastic thymus and cardiac defects were also reported in a considerable number of chimeric mouse fetuses, with a high contribution of human chromosome 21 in mouse somatic cells. Recently, another group has reported a trans-chromosomic mouse model of DS in which tumor angiogenesis is significantly repressed; in particular, *in-vitro* and *in-vivo* angiogenic responses to vascular endothelial growth factor (VEGF) are inhibited by overdose of a set of identified factors transcribed from the three-copy alleles (Reynolds et al., 2010). The evidence shows that some human genes are able to reproduce their physiological functions in mouse cells.

5.5 Functional compensation through additional chromosome segments

As mentioned above, Oshimura and colleagues have developed a large number of HAC applications for biological analysis in the fields of cancer research, DNA repair, telomere research, genomic imprinting, and others. Moreover, they have shown that HAC vector could be used for gene therapy to correct insulin deficiency in mice (Suda et al., 2006) and Duchenne muscular dystrophy (DMD) in mice and in human immortalized mesenchymal stem cells (Hoshiya et al., 2009). The DMD-HAC also functionally replaced the affected allele in a mouse DMD model and in patient iPS cells (Kazuki et al., 2010). Therefore, the combination of patient-specific iPS cells and an HAC containing the responsible genes represents a powerful tool for gene and cell therapies. As previous cases have demonstrated, HAC technology may enable us to create animal models using native human chromosomes.

6. Future research

In the recent successful applications of this technology, human chromosomal regions were transmitted stably to mouse progeny, resulting in the creation of humanized mice for desired chromosomal regions, as mentioned above. An important point to consider is that horizontal chromosome transfer can realize the exchange of genetic material between different strains and species without producing offspring. Combined with chromosome-wide elimination by means of the Cre-inverted loxP system and chromosome transfer technology, the creation of consomic mice derived from different strains might be possible.

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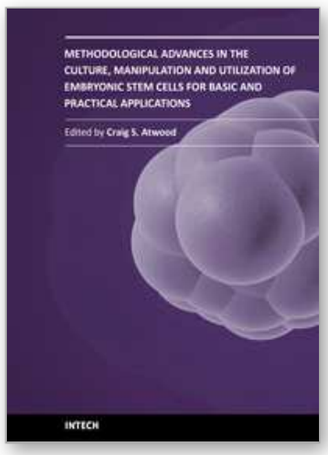

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