

Symposium: Nuclear reprogramming and the control of differentiation in mammalian embryos

Transfer of human artificial chromosome vectors into stem cells



Professor Mitsuo Oshimura DSc is Director of the Research Center for Bioscience and Technology, Tottori University. He has been studying the roles of chromosome and gene mutations in carcinogenesis, with special attention to tumor suppressor genes and cellular senescence, and immortalization. Using microcell-mediated chromosome transfer and chromosome engineering, he is currently producing humanized model mice, for example, human P-450 mouse and Down syndrome model mouse, and construction of human artificial chromosomes for gene therapy and regenerative medicine.

Professor Mitsuo Oshimura

Mitsuo Oshimura¹, Motonobu Katoh

Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, Tottori University, 86 Nishicho, Yonago, Tottori 683-8503, Japan

¹Correspondence: e-mail: oshimura@grape.med.tottori-u.ac.jp

Abstract

Human chromosome fragments and human artificial chromosomes (HAC) represent feasible gene delivery vectors via microcell-mediated chromosome transfer. Strategies to construct HAC involve either 'build up' or 'top-down' approaches. For each approach, techniques for manipulating HAC in donor cells in order to deliver HAC to recipient cells are required. The combination of chromosome fragments or HAC with microcell-mediated chromosome transfer has facilitated human gene mapping and various genetic studies. The recent emergence of stem cell-based tissue engineering has opened up new avenues for gene and cell therapies. The task now is to develop safe and effective vectors that can deliver therapeutic genes into specific stem cells and maintain long-term regulated expression of these genes. Although the transfer-efficiency needs to be improved, HAC possess several characteristics that are required for gene therapy vectors, including stable episomal maintenance and the capacity for large gene insets. HAC can also carry genomic loci with regulatory elements, which allow for the expression of transgenes in a genetic environment similar to the natural chromosome. This review describes the lessons and prospects learned, mainly from recent studies in developing HAC and HAC-mediated gene expression in embryonic and adult stem cells, and in transgenic animals.

Keywords: chromosome transfer, gene delivery, gene therapy, human artificial chromosome, stem cell, trans-chromosomal mice

Introduction

Microcell-mediated chromosome transfer is an important method for mapping a gene to a specific human chromosome when the gene has a special cellular function. For example, this method has been used to map genes involved in recessive genetic disorders, tumour suppression, cellular ageing, metastasis, growth inhibition and DNA repair (Kurimasa *et al.*, 1994; Matsuura *et al.*, 1998; Kugoh *et al.*, 2002, 2003; Lefter *et al.*, 2003; Yamanaka *et al.*, 2004). Several research groups have provided seminal studies on the construction of libraries of microcell hybrids containing a single human chromosome tagged with a selectable genetic marker (**Figure 1**), and on the use of microcell-mediated chromosome transfer to study

gene dosage effects, gene deletions, trans-acting effects and complementation effects (Kaur *et al.*, 1993; Cuthbert *et al.*, 1995; Kadota *et al.*, 2002; Szutorisz *et al.*, 2003; Fukagawa *et al.*, 2004; Kost-Alimova *et al.*, 2004; Upender *et al.*, 2004; Meaburn *et al.*, 2005). A human chromosome tagged with a dominant selectable gene can be transferred to any cell without having to isolate mutant cells lacking the gene. Mouse A9 cells containing a single human chromosome tagged with a dominant selectable gene, *pSV2-neo*, have been constructed (Koi *et al.*, 1989; Kugoh *et al.*, 1999; Tanabe *et al.*, 2000; Inoue *et al.*, 2001). These clones provide chromosome donors for other chromosome transfer experiments (Mitsuya *et al.*, 1999;

Kashino *et al.*, 2001; Meguro *et al.*, 2001; Yoshioka *et al.*, 2001; Okita *et al.*, 2003; Spence *et al.*, 2005; Yanagisawa *et al.*, 2005). Therefore, microcell hybrids provide valuable resources not only for mapping and cloning human genes, but also for functional studies of specific genes and production of animal models (Shinohara *et al.*, 2001; Mizuta *et al.*, 2006; Mukaida *et al.*, 2007).

During the chromosome transfer process, the accidental fragmentation of intact chromosomes can take place and human chromosome fragments (hCF) are thus produced. Low dose irradiation of microcells followed by fusion with recipient cells also induces chromosome breakage, thus resulting in the production of an endogenous functional centromere (Dowdy *et al.*, 1990). The hCF carrying defined contents from the original chromosome are also feasible gene delivery vectors with the use of microcell-mediated chromosome transfer. The transfer of hCF to cells exhibiting various deficiencies has facilitated the mapping and cloning of genes responsible for various defects through functional complementation (Kurimasa *et al.*, 1994; Uejima *et al.*, 1998; Zhu *et al.*, 1998; Cosma *et al.*, 2003; Yawata *et al.*, 2003). Another application of this method is the generation of transgenic animals harbouring hCF with functional human loci (Tomizuka *et al.*, 1997; Shinohara *et al.*, 2000). An advantage of this procedure is that foreign genetic materials can be transferred to mouse embryonic stem (ES) cells by using a human chromosome itself as a vector, which enables the transfer of large segments of genomic DNA, the size of which is far beyond the maximum capacity of conventional cloning vectors such as yeast artificial chromosome (YAC). The introduction of genes accompanied by their cis-regulatory elements enables the physiological expression of the genes in their genomic context with tissue specificity that is difficult to achieve using an expression vector with a transgene.

A problem of hCF vectors is that the structural rearrangement is not predictable and their sequence composition should thus be examined experimentally (Grimes and Cooke, 1998; Brown *et al.*, 2000). A solution to this problem was the creation of chromosome manipulation technologies. When the cloned telomeric DNA sequence (TTAGGG)_n is introduced into the cells, the distal portion of a mammalian chromosome arm is truncated at the integration site by the formation of new telomeres (Itzhaki *et al.*, 1992). The truncation efficiency is improved when the mammalian chromosomes are transferred into the hyper-recombinogenic chicken cell line DT40 (Buerstedde and Takeda, 1991; Dieken *et al.*, 1996; Kuroiwa *et al.*, 1998). Therefore, an endogenous chromosome can be engineered into a vector with well-defined size and sequence. These procedures are known as the 'top-down approach' for HAC formation, in contrast to the 'bottom-up approach' as described below. The recent emergence of stem cell-based tissue engineering has opened up new avenues for gene therapy. The task now is to develop safe and effective vectors that can deliver therapeutic genes into specific stem cell lines and maintain the long-term regulated expression of these genes. This article mainly summarizes recent efforts to use chromosome-based vectors for functional studies, such as top-down HAC-mediated gene expression in various stem cells and animal transgenes, which may have the benefit of overcoming the size constraints of cloned transgenes using other techniques (Basu *et al.*, 2005, 2006; Grimes and Schindelhauer, 2005; Monaco and Moralli, 2006).

Ideal gene delivery vector

To achieve the desired effects, therapeutic genes need to be carried by safe and effective vectors that can deliver foreign genes to specific cells, and thereafter maintain their sustainable expression in a regulated fashion. Prerequisites for the gene delivery vectors are: (i) long-term stable maintenance in host cells without integration to the host genome; (ii) appropriate levels of spatial and temporal expression of therapeutic genes in the specific desired cells; (iii) no risk of cellular transformation or stimulation of the host immune system; and (iv) high transfer efficiency.

Gene therapy as a modern therapeutic approach has been pursued for nearly a decade with viral vectors. A number of different approaches have been attempted to achieve efficient systemic gene transfer and long-term gene expression. Adenovirus-derived vectors have been widely employed in many current gene therapies because of their high infectivity for a wide variety of cell types and tissues, independent of the proliferative state (Russell, 2000; Gugala *et al.*, 2003). However, adenovirus-mediated transgene expression is transient, and it needs high-titre administration. The consequent toxicity and undesired immunological response make adenovirus-based gene therapies risky (Raper *et al.*, 2003). Other popular choices include well-characterized retroviral vectors, which enable sustained transgene expression by the integration of the target genes into host cell genome. However, retroviruses [murine leukaemia virus (MLV)] have been reported to integrate favourably near transcription start regions of the host genome (Wu *et al.*, 2003), thus causing insertional mutagenesis with retrovirus-based vectors (Li *et al.*, 2002). In addition, transcriptional silencing caused by retroviral vectors is often observed in mouse stem cells and transfected haematopoietic stem cells (Challita *et al.*, 1994; Klug *et al.*, 2000). Although early attempts faced many obstacles, with continuous efforts to overcome these concerns, there have been major improvements in some aspects of viral vectors (Lundstrom, 2003). An important issue still remaining is to establish physiological levels of expression and ways of regulating the expression.

Human artificial chromosomes as vectors

A solution to issues raised by the use of currently available viral vector system is provided by the use of human artificial chromosomes (HAC), because they replicate and segregate independently from the host genome as natural chromosomes (Harrington *et al.*, 1997; Guiducci *et al.*, 1999; Grimes *et al.*, 2001; Katoh *et al.*, 2004). HAC are exogenous mini-chromosomes artificially created by either a 'top-down approach' (engineered chromosome), or a 'bottom-up' approach (de-novo artificial chromosome). The chromosomes can then be transferred into other cell lines by microcell-mediated chromosome transfer (MMCT) (**Figure 1**). HAC can faithfully mimic the normal pattern of gene expression because they can hold complete genomic loci, including upstream and downstream regulatory elements (Tomizuka *et al.*, 1997; Ikeno *et al.*, 1998). In addition, because of their episomal existence, many complications, such as silencing of the therapeutic gene or oncogenesis resulting from integration

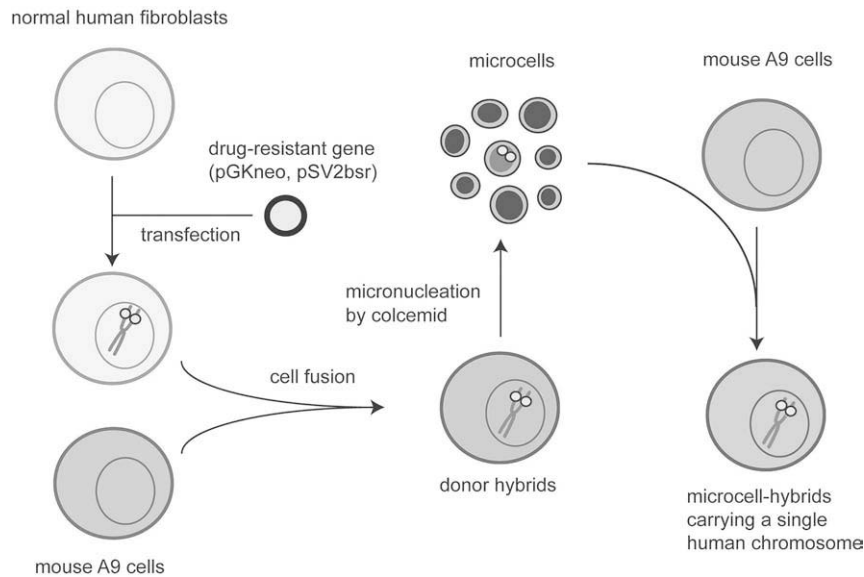


Figure 1. Construction of mouse A9 hybrid cells carrying a single human chromosome by microcell-mediated chromosome transfer. The first step includes marking the human chromosome with a selection marker in the fibroblast and the fusion with mouse A9 cells. The second step is the introduction of the marked human chromosome from the donor hybrid to the recipient A9 cells. The procedure can be divided into several parts: micronucleation of the donor hybrids by colcemid treatment, enucleation in the presence of cytochalasin B, purification of the microcells, fusion with the recipient A9 cells, drug selection of the microcell hybrids, identification of the transferred human chromosome by fluorescence in-situ hybridization and DNA analyses. Libraries of A9 microcell hybrids were constructed containing single human chromosomes.

at unfavourable sites, should be minimized, although there is no proof of this at present. It might also be possible to maintain long-term correction of defective genes because these vectors are stable throughout many cell divisions, at least in human cells (Brown *et al.*, 1996; Katoh *et al.*, 2004; Ren *et al.*, 2005). Such a capability would be advantageous over the current gene delivery methods.

There are other varied types of gene delivery systems that aim to achieve episomal maintenance and introduction of large-sized genomic DNA (>100 kb) (Basu and Willard, 2005, 2006; Grimes *et al.*, 2005; Monaco and Moralli, 2006). They include utilization of: (i) naturally occurring minichromosomes (Conese *et al.*, 2004, 2007); (ii) neocentromere-based minichromosomes (Wong *et al.*, 2002); (iii) alphoid satellite-based artificial chromosome (Lindenbaum *et al.*, 2004); and (iv) self-replicating viral vector amplicons (Black and Vos, 2002; Wang and Vos, 2002; Senior and Wade-Martins, 2005; Hibbitt and Wade-Martins, 2006). Proof of principle in cultured cells has been demonstrated in each approach and applications for mouse transgenesis have been reported in some approaches (Monteith *et al.*, 2004; Wong *et al.*, 2005). The following sections mainly cover and discuss in depth the work on top-down and bottom-up HAC.

De-novo creation of HAC

The initial purpose of generating HAC *de novo* is to improve understanding of the requirements of the chromosome structure and function, particularly the role of the centromere. The

centromere is a complex chromosomal structure providing a distinct mechanical function for the chromosome, which serves as a site for the assembly of the kinetochore and microtubule-mediated chromosome separation during cell division (Sullivan, 2001; Pidoux and Allshire, 2005).

The de-novo assembled HAC was developed in 1997 by Willard and coworkers (Harrington *et al.*, 1997). A combination of human synthetic α -satellite DNA, polymerase chain reaction-generated human telomeric DNA, and randomly shared human-genomic DNA was introduced into a human fibrosarcoma HT1080 cells to assemble an exogenous linear HAC (Harrington *et al.*, 1997). Subsequently, other groups have also reported the successful generation of de-novo HAC using HT1080 cells, on transfection of precursor human α -satellite DNA and telomeres cloned in linear YAC, circular bacterial artificial chromosome (BAC) or P1 phage-delivered bacterial artificial chromosome (PAC) (Figure 2) (Ikeno *et al.*, 1998; Masumoto *et al.*, 1998; Henning *et al.*, 1999; Ebersole *et al.*, 2000; Mejia *et al.*, 2001; Kouprina, 2003; Basu and Willard, 2005; Basu *et al.*, 2005). In most cases, de-novo generated HAC are circular, range from 1 to 10 Mb in size, and have been shown to be mitotically stable. Recently, other systems have been developed to rapidly create bacterial artificial chromosome (BAC)-based HAC using the red-recombination system from bacteriophage λ (Kotzamanis *et al.*, 2005), or using a modified bacterial Tn5 transposon (Basu *et al.*, 2005). Utilization of invasive *Escherichia coli* system may facilitate de-novo HAC formation (Narayanan and Warburton, 2003).

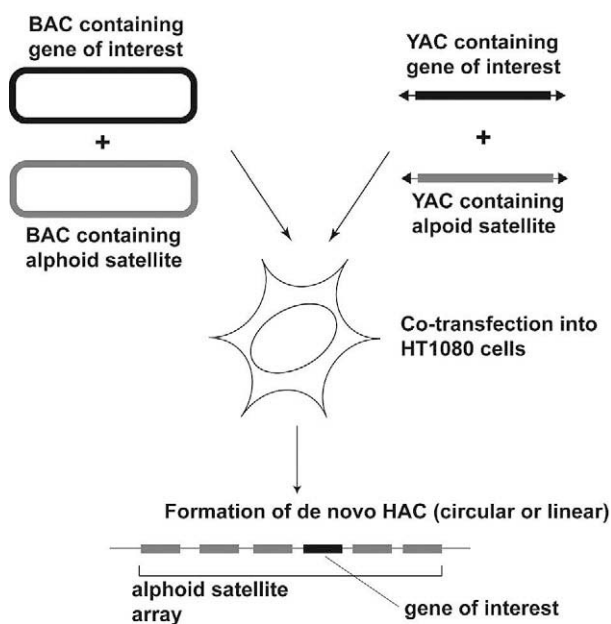


Figure 2. A strategy for incorporating genes into de-novo human artificial chromosomes (HAC). Co-transfection of circular bacterial artificial chromosome (BAC) or linear yeast artificial chromosome (YAC) construct containing alphoid satellite and a drug-resistant gene in the presence of a second circular or linear construct containing a gene of interest has been used to incorporate genes into artificial chromosomes. Following transfection into human HT1080 cells, the clones resistant to the antibiotics are selected and expanded. Multimerization of the input DNA elements reconstructs autonomously replicating artificial chromosomes.

Several studies have demonstrated the feasibility of de-novo generated HAC for the delivery and expression of large human transgenes in human cell lines. The successful functional complementation of hypoxanthine–guanine–phosphoribosyltransferase (HPRT) gene has been observed in HPRT-deficient, human HT1080 cells (Grimes *et al.*, 2001; Mejia *et al.*, 2001). Other studies have shown that the HPRT–HAC are circular and can be maintained at a low copy number in the host cells. These HAC are relatively stable in the absence of selection for several months and functionally complement the metabolic deficiency of host cells. Later, Okazaki and coworkers generated a HAC by cotransfection of a BAC containing either 50 or 100 kb of chromosome 21 α -satellite DNA, with a 180-Kb BAC containing the guanosine triphosphate cyclohydrolase I (*GCH1*) gene (Ikeno *et al.*, 2002). The *GCH1*-HAC was maintained independently in the host cells. The *GCH1* gene expression was induced by interferon- γ , which mimics the regulation of *GCH1* gene expression in the authentic chromosome, thus demonstrating that the transgenes on HAC are subjected to normal regulation. However, several factors limit the application of de-novo generated HAC as gene delivery vehicles. First, the most critical problem is their undefined structure and the unpredictable relationship between the input DNA and resultant HAC, especially in terms of their size and composition. These HAC vary in size, ranging from 1 to 10 Mb, suggesting that complex and unknown rearrangements and/or amplifications occur during the process of mini-chromosome formation. In addition, such HAC often contain unpredicted copy numbers of the gene inserts (Harrington *et al.*, 1997; Ikeno *et al.*, 1998, 2002; Mejia *et al.*, 2001). Second, the input DNA might integrate into the host genome (Harrington, 1997). Third, efficient HAC formation has been successful in limited human cell lines, such as fibrosarcoma HT1080 cells (Moralli *et al.*, 2006). When yeast artificial chromosomes (YAC) containing human alphoid DNA were introduced into mouse LA-9 cells, an extrachromosomal structure was formed but thereafter rapidly

disappeared (Taylor *et al.*, 1996). Recently, substantial progress has been reported in the efficiency of de-novo HAC formation in cell lines other than HT1080 by utilizing HSV-1 amplicon (Moralli *et al.*, 2006). Another technical hurdle hampering the application of de-novo generated HAC is the low efficiency of delivery of these HAC into recipient cells. The direct transfer of the HAC from the donor HT1080 cells into recipient cells is an obstacle (Ikeno *et al.*, 2002). But availability of HSV1-based de-novo HAC formation may facilitate direct formation of de-novo HAC in recipient cells, if it is possible to exclude the dominant cell population in which the input DNA is integrated in host chromosomes.

Engineered creation of HAC

HAC can also be constructed by telomere-associated chromosome fragmentation techniques in a hyper-recombinogenic chicken cell line, DT40 (Buerstedde and Takeda, 1991), although increasing the transferring efficiency to a level similar to the de-novo HAC is a problem that needs to be solved. Such an approach can generate stable, linear mini-chromosomes. Initially, mini-chromosomes ranging in size from 0.5 to 10 Mb have been produced from both the human X chromosome (Farr *et al.*, 1992; Mills *et al.*, 1999; Spence *et al.*, 2002) and Y chromosome (Brown *et al.*, 1994; Heller *et al.*, 1996; Shen *et al.*, 1997, 2000; Yang *et al.*, 2000). These mini-chromosomes retain a normal centromere and are mitotically stable in human cells with only minor rearrangements. These studies have revealed the *cis*-acting DNA sequences required for the centromere function on the human chromosomes X and Y. However, these HAC require a cloning site for inserting exogenous genes.

HAC 21 Δ pq has been constructed by the telomere-directed breakage of human chromosome 21 in DT40 cells (Katoh *et al.*, 2004). To construct this vector, substantial contents of

both the p- and q-arm of human chromosome 21 were whittled down through two rounds of telomere-directed chromosome truncation. Next, a single *loxP* sequence (a target sequence for homologous recombination by Cre recombinase) with a *neo* gene without the promoter was introduced to the remaining q-arm. Therefore, at least in theory, any circular DNA (BAC, plant artificial chromosome (PAC), or circular YAC) with a *loxP* site and a promoter can restore the neo-gene expression by Cre-mediated by insertion at the *loxP* site of the 21Δpq HAC (**Figure 3**). The resulting 21Δpq HAC vector was 4.5 Mb in size, with three useful features: (i) it has well-defined genetic architecture; (ii) it is episomally present independent of the host chromosomes (**Figure 4**); and (iii) it is mitotically stable in human somatic cells and mouse cells, including embryonic stem cells, though relatively less stable in mouse cells. One or multiple copies of a gene of interest can be introduced into the *loxP* site.

A gene coding the enhanced green fluorescence protein (EGFP) introduced into the 21Δpq HAC vector in Chinese hamster ovary cells persistently expressed EGFP, thus indicating that the 21HAC might be useful for a functional study of the transgene *in vitro* (Katoh *et al.*, 2004).

Following this work, several studies using the 21Δpq HAC vector have been reported: (i) the persistent expression of the erythropoietin gene in normal human fibroblasts (Kakeda *et al.*, 2005); (ii) the tetracycline-inducible expression of *DNA-PKcs* gene (Otsuki *et al.*, 2005); (iii) the induction of tissue-specific expression of *EGFP* reporter gene accompanying the in-vitro differentiation in human mesenchymal stem cells (MSC) (Ren *et al.*, 2005); and (iv) exogenous gene expression and antigen-mediated growth regulation of human haematopoietic cells (Yamada *et al.*, 2006).

In the first study, the 21Δpq HAC carrying a single copy of the human erythropoietin (*EPO*) gene was introduced into normal human primary fibroblast (Kakeda, 2005). It remained as an intact single chromosome in each cell and was stably maintained after at least 10–14 population doublings, under non-selective conditions. *EPO* gene expression was maintained for 3 months. *EPO* is a growth factor for erythroid cells that is widely used for the clinical treatment of anaemia associated with chronic renal failure, cancer and the other chronic disease (Goodnough *et al.*, 1997). This study shows the HAC vector to be a potentially useful vehicle for carrying therapeutic genes.

In the second study, the expression of foreign genes in 21Δpq HAC can be efficiently regulated by external signals (Otsuki *et al.*, 2005). In this construct, the *DNA-PKcs* gene is under the control of the tetracycline-responsive elements, and the expression of this gene is regulated by the doxycycline concentration in the media. Next, the *DNA-PKcs* and *neo* genes were placed under a single tetracycline-responsive element with bidirectional promoters to reduce the number of independent transcriptional units. In addition, insulators were inserted at both ends of the tetracycline transcriptional transactivator to separate transcriptional units in order to obtain a higher expression. As expected, the induced expression of *DNA-PKcs* rescued the radiosensitive phenotype of *DNA-PKcs*-deficient Chinese hamster ovary cells.

The 21Δpq HAC vector was also used to mediate cell lineage-specific transgene expression (Ren *et al.*, 2005). A reporter

gene construct with the *EGFP* gene under the control of the osteopontin (OPN) promoter (an osteogenic-specific promoter) was inserted into the 21Δpq HAC vector. The OPN-EGFP/21Δpq HAC vector was then transferred into a human immortalized MSC line, which has the potential to undergo osteogenic, chondrogenic, and adipogenic differentiation. The *EGFP* gene was specifically expressed in the MSC that differentiated into osteocytes in coordination with the transcription of endogenous *OPN* gene, but it was not expressed after adipogenic differentiation induction or in non-induction culture. These results showed that the HAC vector can allow appropriate levels of spatial and temporal expression of loaded genes. This is important because providing appropriate levels of expression for therapeutic genes in association with differentiation under physiological regulation in the specialized cells is particularly important in stem cell based gene therapy.

In another study, a 21Δq HAC containing an *EGFP* reporter gene was introduced into primary haematopoietic stem cells (HSC; Yamada *et al.*, 2006). When the modified HSC were transplanted into sublethally irradiated immunodeficient mice, these HSC retained the ability to differentiate into blood cells and thereafter were still detectable in the bone marrow 2 weeks after the transplantation. To achieve an effective expansion of modified HSC, an antigen-mediated genetically modified cell amplification (AMEGA) system was introduced into the 21Δq HAC (Kawahara *et al.*, 2007). The AMEGA system is a positive screening method for amplifying genetically modified cells using a pair of chimeric receptors of ScFv, which trigger a growth signal in response to a specific antigen, fluorescein-conjugated bovine serum albumin (BSA-FL; Kawahara *et al.*, 2003). The HSC harbouring 21ΔqHAC/ScFvg-gp130 showed a growth advantage after stimulation with BSA-FL, in IL6-dependent cells. These results showed that the HAC vectors combined with the AMEGA system might confer a growth advantage on transduced cells, which thus might be useful for increasing the efficacy of gene therapy *in vivo*.

All applications of the 21HAC-based vectors have shown promise for potential use as gene delivery vehicles. Recently, a 100-kb circular YAC generated by transformation-associated recombination cloning (Kouprina and Larionov, 2006) was successfully introduced into the 21Δpq HAC vector (Ayabe *et al.*, 2005). The HAC vector contains a cloning site, 3' *neo-loxP*, into which circular DNA can be reproducibly inserted by using the Cre-*loxP* system. Fortunately, one of the human PAC/BAC libraries constructed for the human genome sequencing project (Frengen *et al.*, 2000), RPCI-6, has a 5' *neo-loxP* site, allowing the PAC/BAC inserts to be transferred readily into the HAC vector. The RPCI-6 library inserts transferred into the HAC were functional (Kazuki *et al.* in press). These results imply that the physiological expression of the transgene can thus be obtained.

HAC delivery and stability

As described earlier, an important application of artificial chromosomes is their potential use as vectors for gene therapy, although several limitations need to be addressed (**Table 1**). At present, the most commonly used method to introduce HAC into recipient cells is MMCT (Schor *et al.*, 1975; Fournier and Ruddle, 1977; Friedberg *et al.*, 1990; Meaburn *et al.*, 2005). During MMCT, donor cell lines containing a chromosome

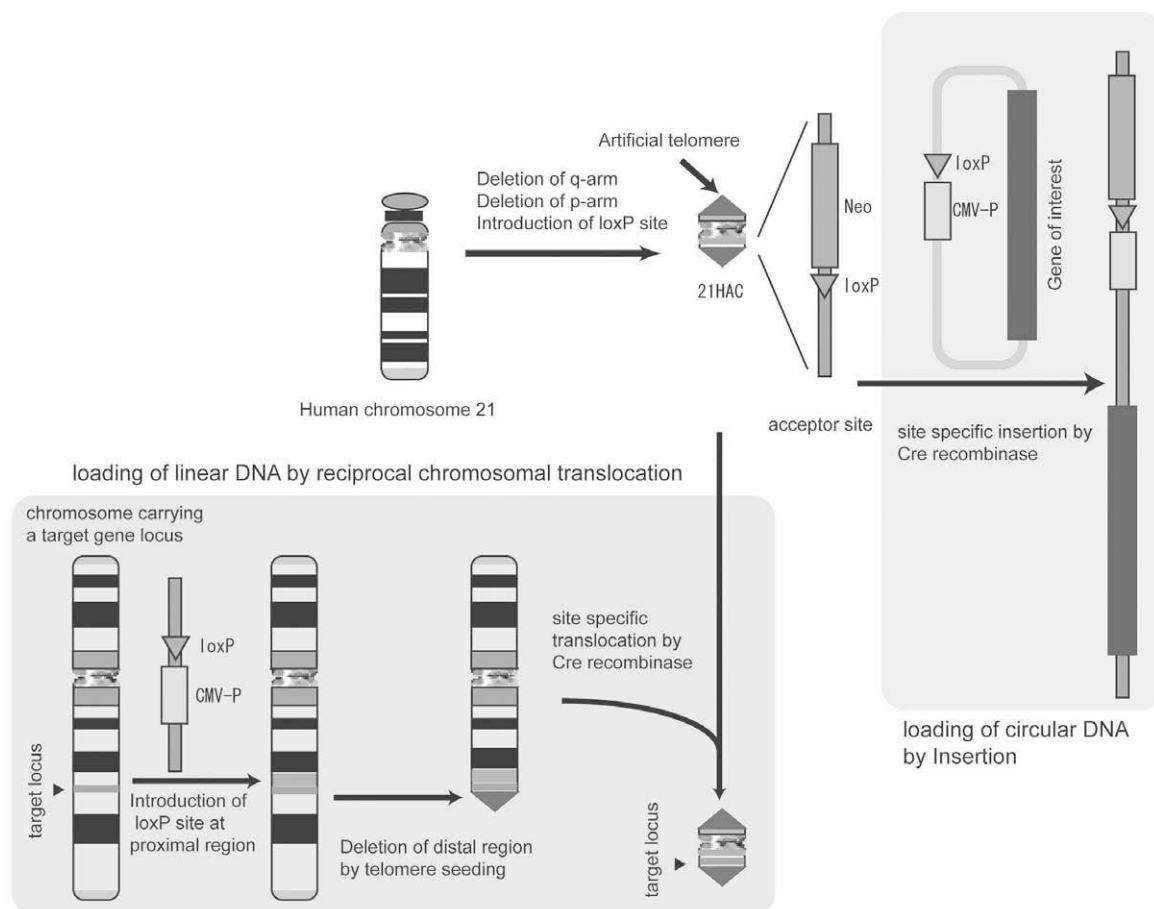


Figure 3. Construction of a human artificial chromosome (HAC) vector from the human chromosome 21 using the top-down approach. The 21HAC is equipped with the *loxP* site for loading a gene of interest. A site-specific recombination event by Cre recombinase is selected by reconstruction of functional *neo* gene, which confers G418 resistance. The gene of interest, isolated in a circular vector, is introduced in the HAC by site-specific insertion. A gene locus spanning megabase size, which is above the capacity of circular cloning vectors, is introduced in the HAC by site-specific reciprocal chromosome translocation. CMV-P = cytomegalovirus promoter.

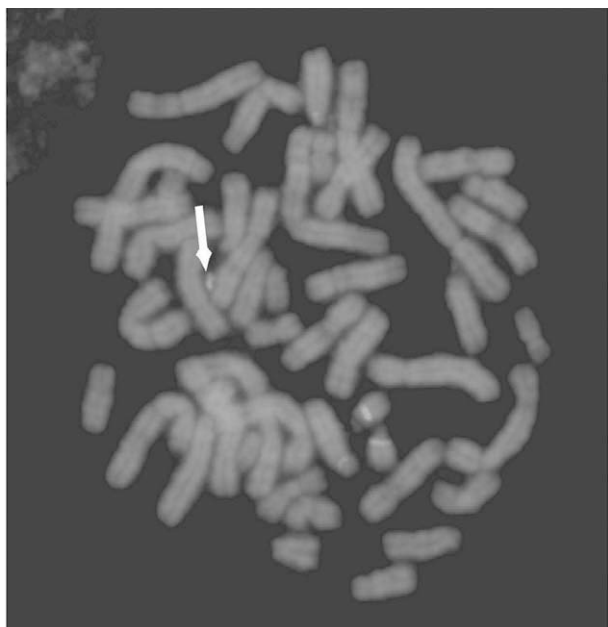


Figure 4. Detection of the 21 human artificial chromosome vector in human fibroblasts by fluorescence in-situ hybridization. Alphoid satellite DNA from human chromosome 21 was used as a probe. A single small chromosome fragment (arrow) was detected in addition to the normal human karyotype. The probe detected the centromere of endogenous chromosome 13 other than chromosome 21.

Table 1. Limitations of engineered human artificial chromosome technology.*Limitation*

Low transfer efficiency to recipient cells *in vitro*.

No available technology for the systemic transfer to cells *in vivo*.

Approximately half of clones isolated after the microcell-mediated chromosome transfer process were structurally rearranged. The effects on the epigenetic signature have not been examined.

with a dominant selectable marker are treated with colcemid to induce the formation of multinucleated cells. Such cells contain numerous micronuclei, each of which has only one or, at most, a few chromosomes. Microcells are then produced from the multinucleated cells by centrifuging at high speeds (4000 g) in the presence of cytochalasin B (**Figure 1**). These microcells are collected and filtered through membranes, and are fused with the recipient cells. The collected microcells contain a heterogeneous group of chromosomes. The cells are then grown under selective conditions where only the hybrid cells containing target chromosome will survive. However, the efficiency of MMCT is very low, in the order of approximately $1 \text{ in } 10^{-6}$ (Katoh *et al.*, 2004; Kakeda *et al.*, 2005).

Another reported method is based on the isolation of the target chromosome from the host cells by flow sorting (flow sorted chromosome transfer, FSCT; de Jong *et al.*, 1999). During FSCT, donor cells are first treated with iodo-deoxyuridine and then are blocked at mitotic metaphase by treatment with colchicine. These chromosomes are then stained with Hoechst 33258 and chromomycin A3 and then harvested and purified from the background of the host chromosomes. The efficiency of FSCT (transfer of 60 Mb of HAC) is higher than MMCT ($1 \text{ in } 10^{-6}$ to 10^{-4}) (de Jong *et al.*, 2001).

Both MMCT and FSCT have been adopted to transfer a single, intact chromosome from donor to recipient cells. MMCT has a lower risk of truncation or rearrangement of the transferred chromosome (Klobutcher *et al.*, 1980), and FSCT is more efficient. However, the transfer of smaller sized HAC (approximately 10 Mb) has not yet been examined.

Another concern regarding gene delivery vectors is mitotic stability in host cells. The stable maintenance of the engineered HAC has been reported in immortalized human cell lines, mesenchymal stem cells, and primary fibroblasts (Katoh *et al.*, 2004; Kakeda *et al.*, 2005; Ren *et al.*, 2005). Several experiments have shown that the HAC seem to be more stable in human cells rather than in rodent cells (see the next section for the in-vivo stability of HAC). The reason needs to be clarified in the future. On the other hand, the stability of de-novo HAC has been proven in a few cell lines including HT1080 (Moralli *et al.*, 2006), but it has yet to be explored in other human cells.

Stability of the gene delivery vector in somatic cells consists of three criteria; copy number maintenance during mitosis, structural integrity during mitosis, and persistent regulated expression of an introduced gene in descendant cells. Most

studies describing artificial chromosomes to date have merely mentioned the retention rate during long-term culture. To evaluate the utility of various types of vectors, the other two criteria should be addressed. For validation of reproducible expression, loading a gene as internal standard to the HAC vector may be useful. *Chuk*, also known as Ikb kinase α , which shows a consistent expression level during preimplantation development (Falco *et al.*, 2006), is a candidate for an internal standard.

Trans-chromosomal mice

Another important application of HAC or hCF is animal transgenesis (**Figure 5**). The ability of hCF to act as vectors for introducing large stretches of human DNA into mice was first demonstrated in 1997 (Tomizuka, 1997). The hCF containing the human immunoglobulin gene locus was introduced into mouse ES cells, followed by the production of chimeric mice. Transferred hCF were stably maintained as an extra chromosome in the somatic cells of mice and their human genes were expressed under proper tissue-specific regulation. In some cases they could be transmitted through the germline, resulting in the establishment of novel mouse strains [trans-chromosomal (Tc) mice] containing a heritable hCF (Tomizuka *et al.*, 1997, 2000). Therefore, employing chromosome vectors to create transgenic animals may be useful for overcoming the size constraints of cloned transgenes in conventional techniques and to facilitate functional studies of the human genome.

The stability of hCF in interspecies hybrids may be affected by many factors, such as the centromere sequence and the genetic background of host cells. The importance of the centromeric alphoid DNA sequences is evident from a study of the different stabilities of human-engineered chromosomes in various host cells. Human acrocentric chromosomes 13, 14, and 21 have been reported to share four different subfamilies of α -satellite DNA, whereas chromosomes 15 and 22 do not. Chromosome 14 has another specific alphoid subfamily (Vissel and Choo, 1991). The in-vivo and in-vitro stability of human chromosome fragments showed that the hCF14 (SC20) are more stable than the hCF2 (2-W23) or hCF11 in mouse ES cells, cultured human tumour cells, and chicken DT40 cells (Shinohara *et al.*, 2000). The germline transmission of hCF has been observed in chimeras from ES cells containing hCF2, 7, 14 or 21, but not hCF22 (Tomizuka *et al.*, 1997, 2000; Shinohara *et al.*, 2000; Kazuki, 2001). These observations suggest that the specific alphoid subfamily might contribute to the stable

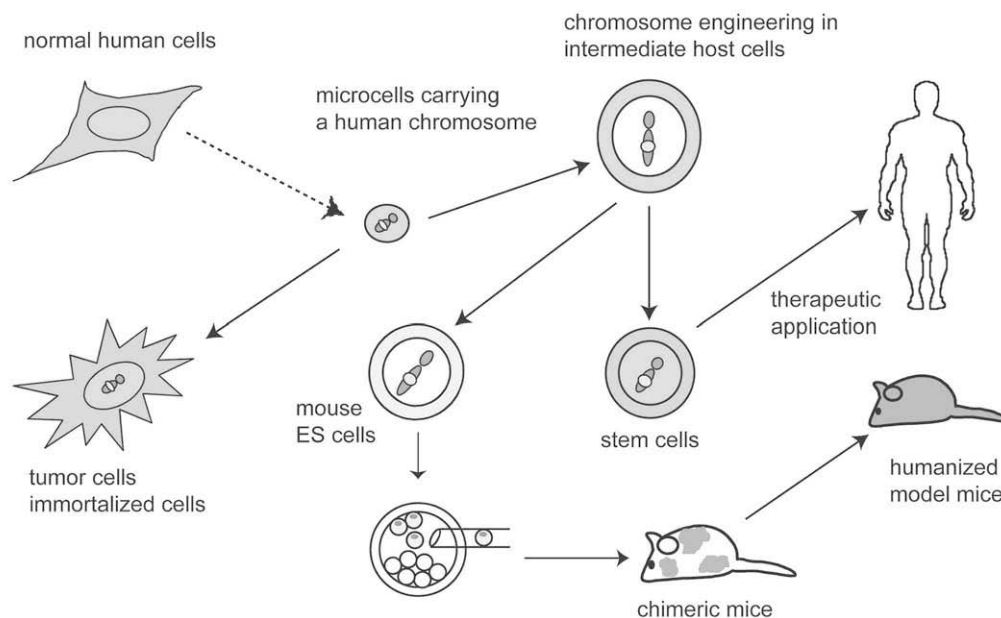


Figure 5. The application of chromosome engineering and its products. Chromosome engineering techniques, which are based on chromosome transfer, are applicable for gene therapy, regenerative medicine, the production of humanized model animals, and the production of therapeutic substances in transchromosomal livestock for industrial purposes. ES cells = embryonic stem cells.

maintenance of the hCF. Other studies have shown that human Y chromosome fragments are stable in human and chicken cell lines but not in mouse cells (Shen *et al.*, 2001). However, one of the mini-chromosomes (ST1) obtained mouse centromeric DNA fragments and was mitotically stable in mouse cells and transmitted to a germline (Shen *et al.*, 2000). Alternatively, the genes located on the hCF might influence chromosome stability. The genetic background of mice also influences the stability of hCF. The C57BL/6 strain yielded a higher retention rate than the mouse MCH strain (Kazuki *et al.*, 2001). In general, the introduced hCF were transmitted more efficiently to a chimeric female germline than to a male, thus reflecting possible differences between the sexes during meiosis and mitosis (Voet *et al.*, 2001).

As mentioned above, one major problem of hCF vectors has been the fact that they are structurally undefined (Brown *et al.*, 2000; Grimes *et al.*, 2001). The hCF are usually generated as a consequence of accidental fragmentation of intact chromosomes during the chromosome transfer process and they therefore run the risk of containing unrelated, deleterious genes in addition to the genes of interest. To address this problem, a 'chromosome cloning' technique was recently developed that enabled the construction of HAC that included only defined chromosomal regions in the homologous recombination proficient chicken DT40 cell line (Kuroiwa *et al.*, 2000). The complete human genome sequence, which is now available, should facilitate the generation of HAC containing only desired chromosomal regions as mentioned earlier.

The successful introduction of hCF into mouse ES cells and the generation of chimeric mice have opened a new avenue in animal

transgenesis (Kuroiwa, 2000; Tomizuka *et al.*, 1997, 2000). Using this chromosome-cloning technique, various human chromosome regions can be cloned into a minichromosome or HAC vector by homologous recombination. A minichromosome derived from 14 (SC20), which contains immunoglobulin IgH loci, was chosen as a basal vector because of its relatively high mitotic and meiotic stability in mice (Shinohara *et al.*, 2000; Tomizuka *et al.*, 2000). A mitotically unstable human chromosome fragment hCh22 containing the 10 Mb Igλ-light chain locus was cloned into the SC20 to generate λHAC. The λHAC was transferred into the mouse ES cells to generate transchromosomal (Tc) mice. When immunized with human granulocyte colony-stimulating factor (G-CSF), both the humanized Igλ and IgH were functionally expressed in chimeric mice. This study clearly shows the possibility of expressing human antibodies in mice. This technology was applied for cows with the HAC in combination with nuclear transfer as well (Kuroiwa *et al.*, 2002a,b).

Another application of chromosome engineering technology is to generate model animals of human aneuploidy syndromes, which are caused by extra dosage of wild-type genes on human chromosome. Trisomy of chromosome 21 is the most common live-born human aneuploidy and results in a constellation of features known as Down syndrome (Antonarakis *et al.*, 2004). Two groups have successfully generated Tc Down syndrome model mice. Shinohara and coworkers generated the first Tc mouse by introducing a human chromosome 21 fragment (hCF21), approximately 45 Mb in size, into the mouse ES cells (Shinohara *et al.*, 2000). The hCF21 was transmitted to the mouse germline to at least the F3 generation (Kazuki *et al.*, 2001). The chimeric mice showed cardiac abnormalities

and behavioural impairment similar to patients with Down syndrome (Shinohara *et al.*, 2001). However, the retention rate of the hCF21 was variable among individual mice, for example, 21–92% in brain and 10–92% in tail fibroblasts.

According to the findings of these studies, Fisher's group applied the same approach to generate an aneuploid mouse that stably transmits a freely segregating, almost complete human chromosome 21 (O'Doherty *et al.*, 2005). The retention rate of human chromosome 21 is 55% in the spinal cord, 24% in the spleen, 66% in brain nuclei, and 49% in the spleen. This mouse line manifests the symptoms similar to Down syndrome in humans. Tc mouse technology can be useful to dissect other human aneuploidies as well as to identify and map genes that contribute to the aneuploidy diseases.

Furthermore, the hCh7 region around the CYP3A locus was cloned into the SC20 vector. Transcript of cytochrome P450 3A4 (CYP3A4) gene was also detected specifically in liver and small intestine of the SC20-transferred chimeras, which coincides well with the tissue specificity observed in human (Kuroiwa *et al.*, 2002a,b; Kazuki, unpublished data). The availability of structurally defined HAC vectors would be of great value in the construction of animals carrying human genetic elements to model specific diseases or the production of various therapeutic products for industrial purposes.

Prospects and conclusion

A potential application of the HAC vector may be its use in the treatment of human genetic diseases (Figure 6). Prior to the use of HAC vectors in the clinical treatment of patients, their efficacy and safety as a treatment modality should be evaluated in animal models. Indeed, the advantages of chromosome

vector system in animal transgenesis, mentioned above, are also complementary and desirable characteristics for therapeutic vectors to overcome various problems in existing viral and non-viral vector systems, although the in-vivo delivery to cells is currently not available. For example, dystrophin is the largest gene (2.4 Mb) in humans and is responsible for Duchenne muscular dystrophy (Dunne and Epstein, 1991). Because of its large size, the entire dystrophin locus has never been cloned, even using YAC vectors. At least in theory, this gene can be cloned into a HAC. As a result, the expression of the full-length dystrophin could be used in gene therapy. HAC also have the advantages of being mitotically stable in the absence of selection and they have an indefinite cloning capacity, thus allowing for the insertion of all control elements for the correct expression of the transgene. However, as a result of their large size, they are difficult to handle and can only be recovered in small quantities. Future research will focus on the efficient delivery of such vectors to the cells.

Stem cells possess two characteristic features: the ability for self-renewal, and the ability for multilineage differentiation. Since the first discovery of haematopoietic stem cells in mice (Till and McCulloch, 1961), there have been significant advances in the identification, purification, in-vitro expansion and genetic manipulation of various types of stem cells (Galli *et al.*, 1993; Vogel, 2000; Quesenberry *et al.*, 2002; Mayani, 2003; Preston *et al.*, 2003; Shufaro and Reubinoff, 2004; Fruehauf and Ho, 2005; Keller, 2005). Specifically, the bone marrow-derived MSC have drawn attention as a potential progenitor cell source for repair and regeneration of diverse adult tissues, including fat, cartilage, bone marrow stroma, and skeletal muscle. In comparison to pluripotent ES cells, MSC are preferable for therapeutic purposes (Tuan *et al.*, 2003) because they do not cause as much legal and moral controversy and they can be more easily directed to specific lineages than

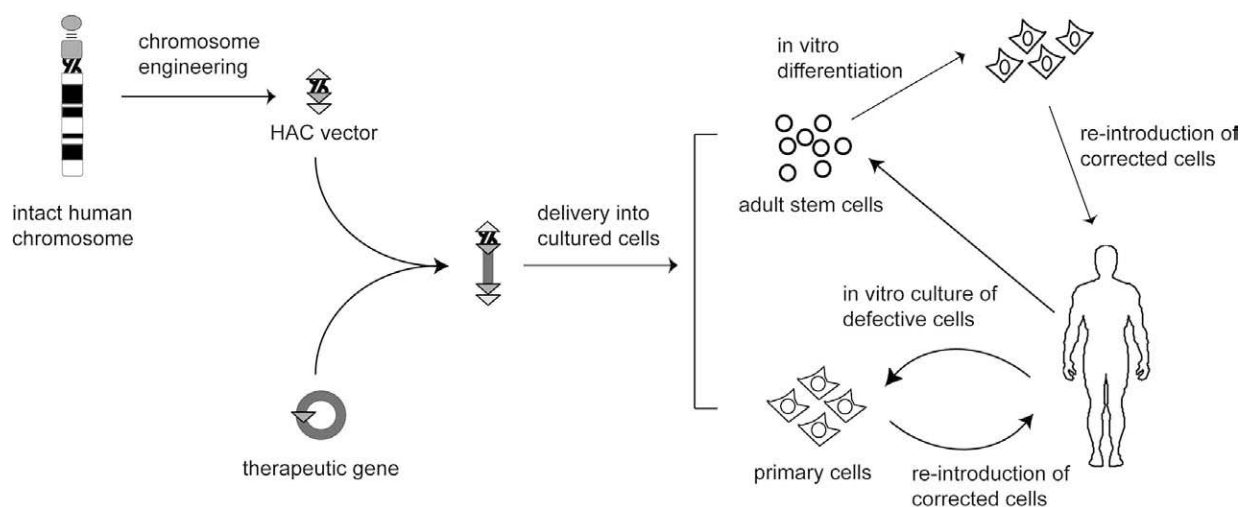


Figure 6. The use of human artificial chromosomes (HAC) for ex-vivo gene therapy. Cells are taken from a patient and cultured *in vitro*. A human artificial chromosome vector containing a therapeutic gene is then transferred into these cells. Genetically corrected cells are thereafter reintroduced to the patient. Possible targets of gene delivery are adult stem cells or primary cells such as fibroblasts. Adult stem cells carrying the therapeutic vector are first allowed to differentiate into the desired cell type and are then reintroduced to a patient.

the ES cells. In addition, because they are obtained from the patient's own tissues, these cells can avoid potential immune rejection. Lastly, engineered adult MSC can potentially repair the damaged tissue *in vivo* (Chapel *et al.*, 2003).

Since the successful construction of functional HAC, substantial efforts have been made to use them to deliver genes to various stem cells, including mouse ES cells and tissue-specific human stem cells. The transfer by HAC of multiple lineage and stage-specific gene promoters, followed by fluorescent protein coding gene, may facilitate the isolation of defined cell populations at spatiotemporal context during embryogenesis or tissue differentiation. *Chuk*, also known as I κ B kinase α , which shows a consistent expression level during preimplantation development (Falco, 2006), may be useful as an internal standard. A feasible therapeutic application of the HAC vectors would be the ex-vivo transduction of a gene of interest by HAC into stem cells followed by autologous transplantation (Figure 6). As the size of the HAC was reduced, it no longer contained any extra-human genes, thus increasing its value as a vector for human gene therapy. A remaining challenge is developing methods that will allow for the efficient delivery of these very large molecules into patient-derived stem cells with a limited life span. Most recently, several research groups have developed a technology to induce ES-like stem cells, so-called induced pluripotent stem (iPS) cells, from adult mouse fibroblasts (Takahashi and Yamanaka, 2006; Maherali *et al.*, 2007; Okita *et al.*, 2007; Wernig, 2007). Once replicated with human cells, it may be utilized as a potential material for gene corrections. HAC vector accommodating the four initiation factors (Oct4, Sox2, c-Myc and Klf4) and drug-resistant marker gene under the regulation of pluripotent marker genes (such as *Nanog*) might be suitable for development of iPS cells.

Although substantial improvements in the delivery methods are expected, the feasibility of producing patient-specific pluripotent cells to produce multipotent stem cells is already evident from recent studies and although still in its infancy, this technology is expected to continue to develop in parallel with HAC technology.

Note added in proof: During preparation of this article, two reports have shown the establishment of iPS cells from adult and fetal human fibroblasts (Takahashi *et al.*, 2007; Yu *et al.*, 2007). These advances could enable the creation of patient-specific stem cells, which would facilitate the gene therapy study.

Acknowledgements

This study was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the 21st Century COE Program, The Research Core for Chromosome Engineering Technology, and a grant from the Ministry of Health, Labor and Welfare. We also thank Mr Yu-ichi Iida and Mr Donglai Qi for preparing the manuscript. The authors report no financial or commercial conflicts of interest.

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Received 25 June 2007; refereed 3 August 2007; accepted 26 October 2007.