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TransChromo Mouse™

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Introduction

Various techniques for producing chimeras from genetically manipulated mouse ES cells have provided valuable tools for studying gene functions *in vivo* and developmental regulation of gene expression. A variety of procedures, such as targetted modification of specific genes by homologous recombination (Mansour *et al.*, 1988), introduction of cloned exogenous DNA fragments (Jakobovits *et al.*, 1993; Strauss *et al.*, 1993) and chromosome manipulation by the Cre-*lox*P system (Ramiez-Solis *et al.*, 1995; Smith *et al.*, 1995) have facilitated the genetic manipulation of ES cells. Cell fusion, or chromosome transfer, is an indispensable tool for characterizing gene expression in somatic cells (Hooper, 1987). Although several such studies using embryonic carcinoma (EC) cells (Benham *et al.*, 1983) have been reported, the techniques described have rarely been used to manipulate embryonic stem (ES) cells. The creation of animals manifesting specific foreign chromosome-mediated functions using transchromosomic ES cells allows the study *in vivo* of very large genes or gene clusters that far exceed the limited length of DNA that can be cloned by conventional techniques. Because of their large size, these HACs allow the inclusion of critical remote regulatory elements and thereby permit us to reproduce the conditions necessary for the proper expression of the transchromosomic elements. This approach opens attractive possibilities for constructing animal models representing human genetic diseases involving chromosome abnormalities.

More than 20 years ago, Illmensee and co-workers reported the production of viable chimeric mice from hybrid mouse EC cells fused with human fibrosarcoma or rat hepatoma cells (Illmensee *et al.*, 1978; Illmensee and Croce, 1979). However, they

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Abbreviations: ES cells, embryonic stem cells; EC cells, embryonic carcinoma cells; FACS, fluorescent activated cell sorting; FISH, fluorescent *in situ* hybridization; GCSF, granulocyte colony-stimulating factor; GFP, green fluorescent protein; HAC, human artificial chromosome; hChr, human chromosome; HSA, human serum albumin; Ig, immunoglobulin; Ig-H, immunoglobulin heavy chain; Ig-κ, immunoglobulin κ light chain; MMCT, microcell mediated chromosome transfer; MH(ES) cells, microcell hybrid ES cells; TC, transchromosomic or TransChromo.

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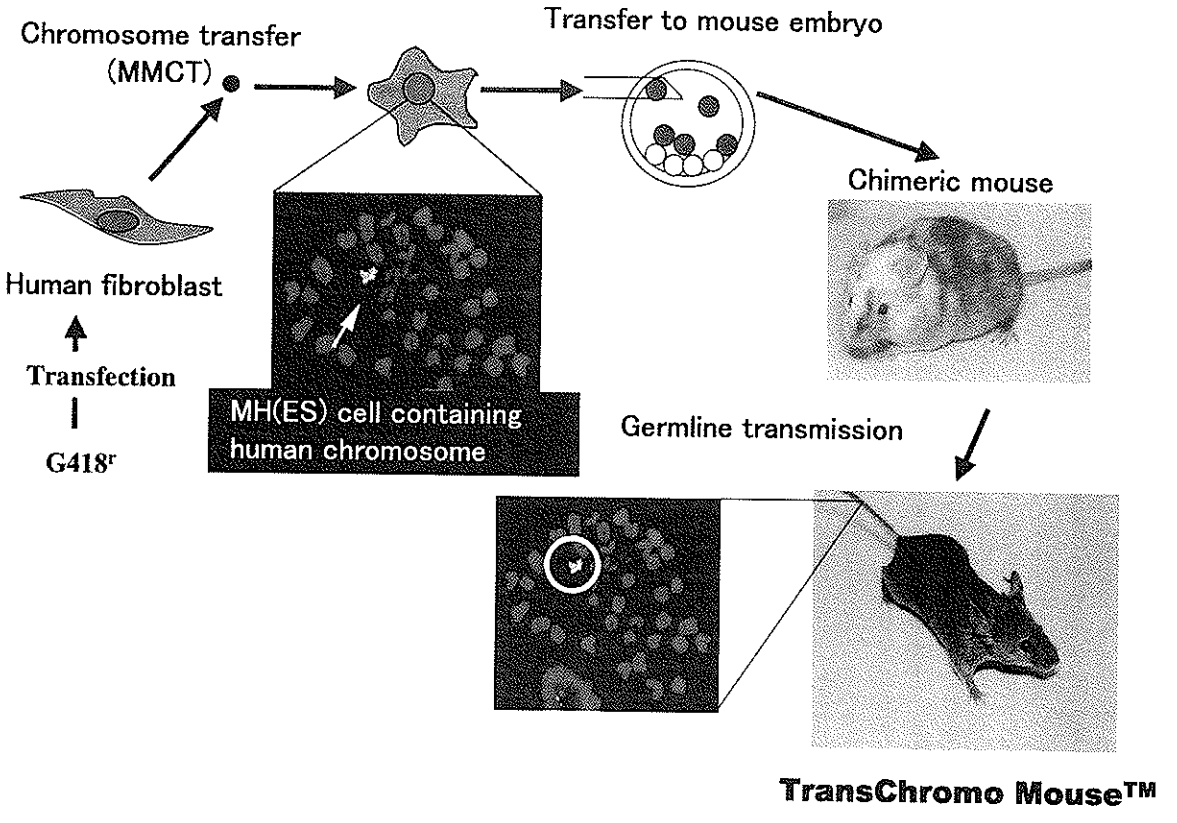

Figure 3.1. A schematic diagram showing the construction of MH(ES) cells to express human genes on the transferred chromosomes (Ishida *et al.*, 1998). G418^r: G418-resistant gene.

could not unequivocally demonstrate that the hybrid EC cells had contributed to normal tissues of the mice. It has generally been supposed that extensive genetic modification, such as cell fusion or chromosome transfer, causes loss of pluripotency. Consequently, until our studies, the generation of animals from cells that have undergone extensive genetic modification is an issue that has escaped rigorous examination.

In this paper, we review the use of a novel procedure that allows the introduction of very large pieces (> 1 MB) of foreign genetic material into mice by using a chromosome itself as a 'vector'. The power of this approach was demonstrated by the derivation of the first examples of 'trans-chromosomic (TC) mice or TransChromo (TC) Mouse™' containing a heritable foreign chromosome (Tomizuka *et al.*, 1997). The method was applied to the creation of TC mice containing fragments of human chromosomes (hChrs) 14 and 2 bearing the immunoglobulin heavy (Ig-H) and (Ig-κ) loci respectively and for expressing fully human antibodies (Tomizuka *et al.*, 2000). Further investigation has shown that, while various hChr fragments could be introduced into mice via ES cells, the hChr 14 fragment employed displayed superior retention to that of other chromosome fragments, such as those derived from hChr 2 and hChr 22. A new and powerful cloning procedure was used to improve the retention and transmission in mouse cells of hChr fragments that are not retained well. This cloning strategy combines Cre/*lox*P-mediated chromosome translocation with teleomere-directed chromosome truncation in homologous recombination-proficient chicken DT40 cells (Kuroiwa *et al.*, 1998, 2000). This system was used to clone inserts in excess of 1 megabase from a poorly-retained hChr into a stably-retained hChr 14 fragment-based mini-chromosome vector.

Production of chimeric mice retaining hChrs

The strategy developed in our laboratory for introducing a hChr into mice is outlined in *Figure 3.1*. The pSTneoB, G418-resistant gene (G418^r), whose promoter was active in ES cells, was transfected into human fibroblast cells. A pool of G418^r human fibroblast cells was fused with A9 mouse cells to prepare microcells, which were then fused with A9 cells to effect chromosome transfer and selected with G418 in order to obtain a library of A9 clones containing various pSTneoB-tagged human chromosomes or chromosome fragments. This library, including about 700 independent clones, was screened with PCR primer pairs specific to a human chromosome to identify A9 clones containing the hChr fragment of interest. Microcells prepared from these A9 clones were transferred to mouse ES cells (TT2: 40, XY or TT2F: 39, XO). The resultant MH(ES) cells were injected into 8-cell stage embryos of albino MCH(ICR) mice, and the injected embryos were then transferred into the uteri or oviducts of pseudopregnant mice. A chimeric mouse could be identified by its agouti coat colour. The percentage of agouti coat colour provides an indicator of the extent of MH(ES) cell contribution to the chimera.

MMCT was developed by Fournier and Ruddle (1977) more than 20 years ago and has been used extensively as a tool for characterizing gene expression in somatic cells. However, it has been generally supposed that extensive genetic modification, such as chromosome transfer or fusion with another cell, causes a loss of pluripotency. Consequently, MMCT has never been used in conjunction with ES cells to produce

hChr-bearing chimeric mice. In spite of our initial concern, based on the coat colour of resultant offspring, we found – and reported – that all tested MH(ES) clones transchromosomic for hChr fragment 4, 2, or 22 had the ability to contribute to viable chimeras (Tomizuka *et al.*, 1997). Furthermore, except for the fact that males derived from the hChr 14 fragment were sterile and had small testes, chimeric animals were essentially free of recognizable physical abnormalities. Various tissues were taken from chimeric animals and examined by Southern blot analysis using an L1 probe and by glucose phosphate isomerase isozyme analysis. Southern analysis revealed the presence of the hChr fragment in all tissues examined. The isozyme analysis demonstrated that retention rate of the transchromosome was comparable with contribution of the ES cells in the tissues. The presence of the transferred human chromosome in somatic cells was also confirmed by FISH analysis of primary tail fibroblast cultures derived from chimeric animals. Interestingly, in the B cell lineage of these chimeras, human antibody genes on the transferred hChr fragments undergo rearrangement and are transcribed properly to express functional human antibodies. In addition, examination of the expression of other human genes on the hChr fragments showed that they too were expressed only in appropriate tissues and therefore were under proper tissue-specific transcriptional regulation in the chimeras.

TransChromo Mouse™

The chimeras retaining hChr fragments were bred with albino MCH(ICR) mice to determine if the MH(ES) cells contribute to the germline. Agouti offspring were born from chimeras retaining hChr 2 or 14 fragments (Tomizuka *et al.*, 1997, 2000). The sizes of the hChr 2 and 14 fragments were found by these researchers to be between 10 and 20 Mb, as shown in *Figures 3.2A* and *3.2B*. The transmission efficiency of the hChr 14 fragment is about 33% in males and females and that of the hChr 2 fragment is about 25% in females and 10% in males. These actual frequencies contrast with a theoretical frequency of 50% since the hChr has haploid rather than diploid representation. We have named mice containing heritable foreign chromosome fragments as 'TransChromo (TC) Mouse™'. A breeding programme involving the two lines of TC mice (TC-Chr 14 and TC-Chr 2) and two lines of mice, one of which is Ig-H KO and the other, Ig-κ KO, led to successful production of the double TC/mouse IgKO mice (*Figure 3.3*). Examination of a culture of tail fibroblasts derived from the double TC/IgKO mouse by FISH demonstrated the presence of cells that retained both hChr 14 and 2 fragments (*Figure 3.3*). The double TC/IgKO mouse expresses human Igs in the sera at levels that are comparable to the levels of mouse Ig present in the serum of normal mice. Notably, it was found that the antigen-specific titre of human antibody increased upon immunization with human proteins, such as human serum albumin (HSA) and human granulocyte colony-stimulation factor (GCSF).

Stability of the hChr fragment *in vitro* and *in vivo*

Metaphase spreads of tail fibroblasts prepared from TC mice retaining hChr fragments 14 and 2 had been examined by FISH to evaluate the retention of hChr fragments in somatic cells. About 78% and 30% of the spreads retained hChr 14 and 2, respectively. Based on its almost perfect (data not shown) mitotic transmission in

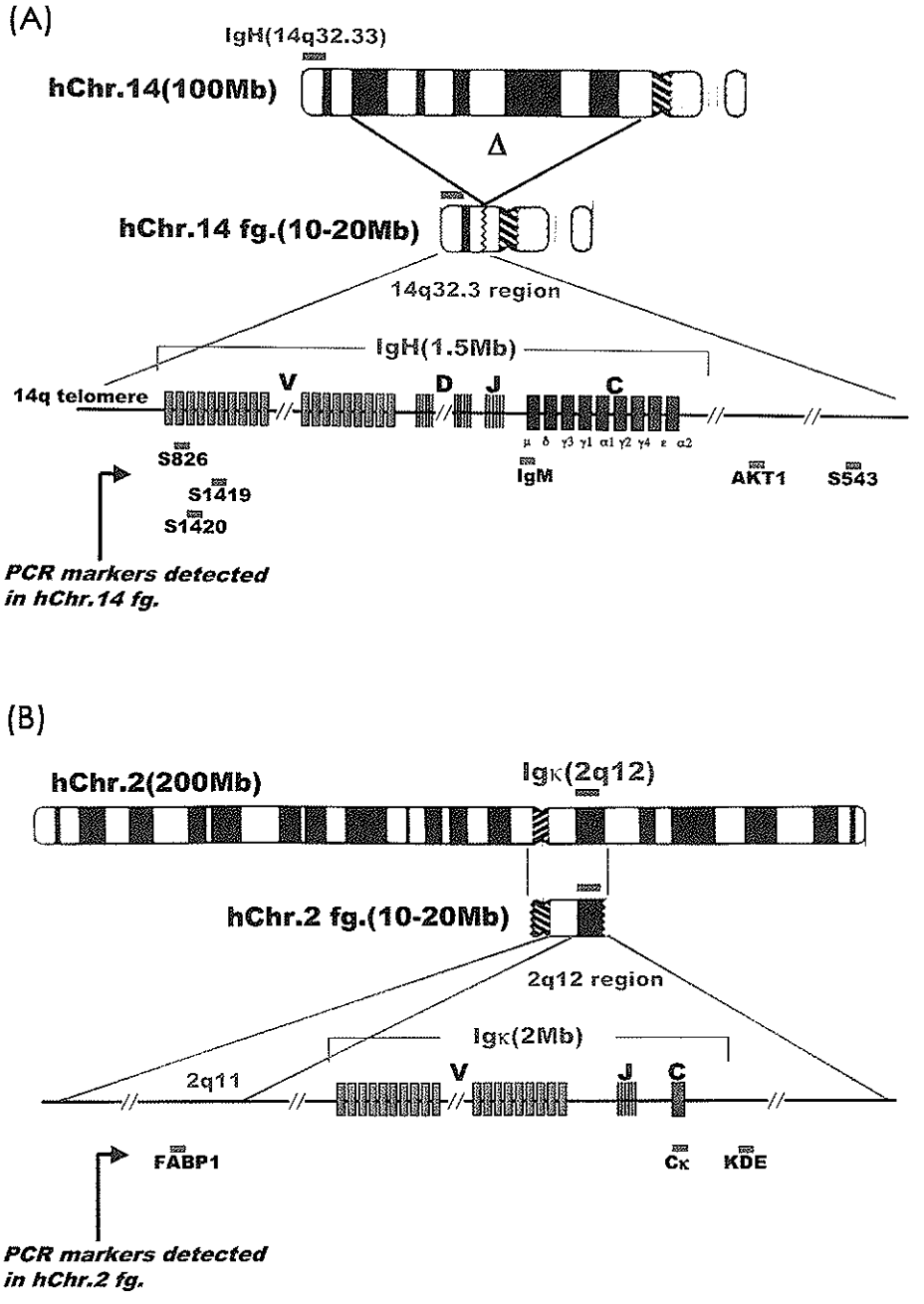

Figure 3.2. The human chromosome fragments transmittable through germline in mice. A) the hChr 14 fragment had a large interstitial deletion from the original hChr 14; B) the hChr 2 fragment had large truncations at both of the short and long arms. Existence of the several genes on the hChr 14 or the hChr 2 fragment was determined by PCR analysis (Ishida *et al.*, 2002).

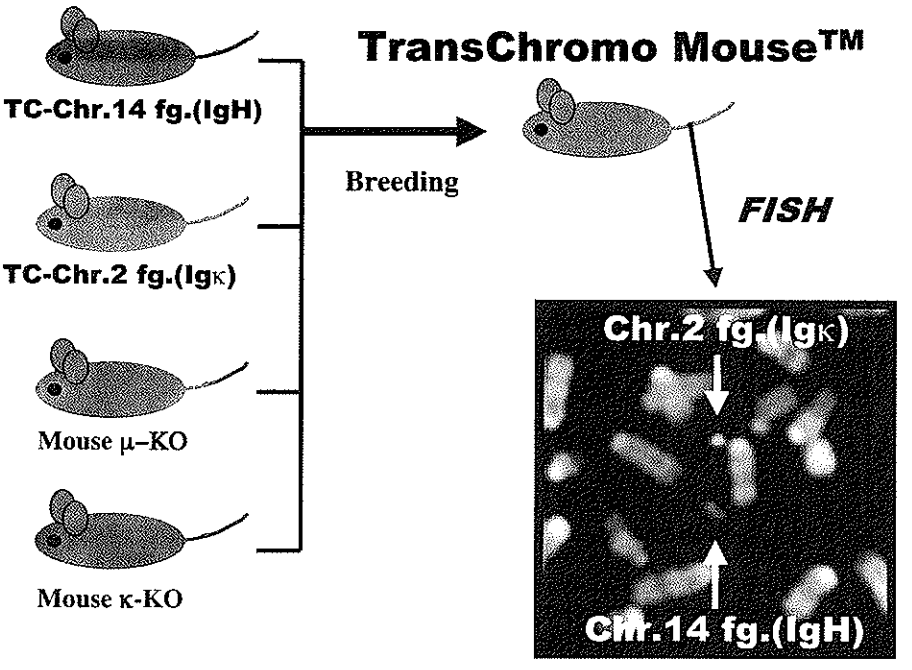

Figure 3.3. Generation of TC Mouse™. The hChr 14 fragment-retaining TC Mouse, the hChr 2 fragment-retaining TC Mouse, the endogenous mouse μ chain KO mouse and the endogenous mouse κ chain KO mouse were crossbred. Finally, the double TC/Ig-KO mice, which retained both of the hChr 14 and hChr 2 fragments and whose endogenous mouse μ- and κ chains were homo-KO, were obtained. Two-colour FISH analysis of tail fibroblast metaphase spreads prepared from a 4-week-old double TC/Ig KO mouse (Tomizuka *et al.*, 2000).

MH(ES) cells, a high level of transmission of the hChr 14 fragments in mice was expected. Similar observations (data not shown) in MH(ES) cells led us to expect that hChr 2 would have a lower frequency of transmission *in vivo*. In this regard, we have found that the transmission of a hChr 22 fragment encoding λ, another human light chain, was not high in either mice or ES cells.

Improvement of instability of the hChr

It was necessary to achieve high rates of transmission for the hChr fragments encoding human light chain genes in order to obtain higher expression level of human Igs in the TC mouse and more variety of hybridoma clones from the immunized TC mouse. An immediate solution to the problem was to crossbreed the TC mouse retaining stable hChr 14 fragment encoding human Ig-H gene locus with the transgenic mouse containing human Ig-κ gene mini-locus, which encodes about a half of the duplicated human Ig-κ valuable region segment clusters and is stably integrated into the mouse genome (Fishwild *et al.*, 1996; Mendez *et al.*, 1997). More recently, we have successfully produced the crossbred mouse which shows the much higher performance in hybridoma production by crossbreeding the hChr 14-TC mouse (whose endogenous mouse Ig-H and κ are KO) with the human Ig-κ transgenic mouse (whose endogenous mouse Ig-H and κ are KO) (Ishida and Lonberg, 2000).

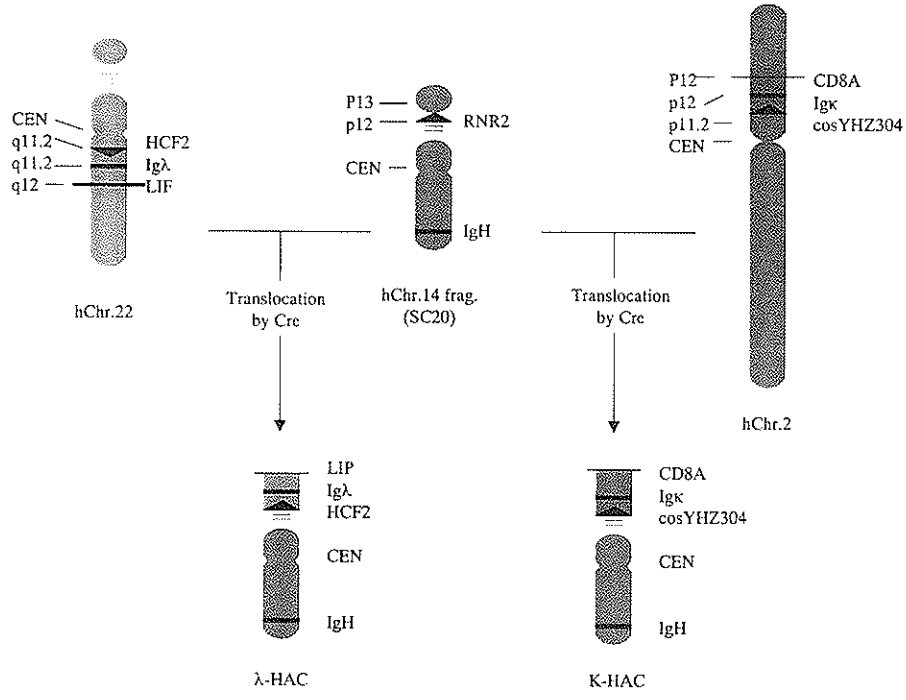

Figure 3.4. The whole scheme of the system. DT40 cells containing the IgH locus-bearing hChr 14 fragment, a potential human mini-chromosome vector, were transfected with the targetted *lox*P insertional vector to integrate a *lox*P sequence to *RNR2* locus at 14p12 to make a cloning site for chromosome inserts. The chromosome insert containing Ig-light chain λ locus derived from hChr 22 was prepared by truncation of hChr 22 at *LIF* locus and insertion of a *lox*P sequence at *HCF2* locus in DT40 cells. The region between *HCF2* and *LIF* loci containing Ig λ locus on hChr 22 is cloned into the *RNR* locus on the short arm of the hChr 14 mini-chromosome vector by Cre-recombinase mediated translocation in DT40 cells. Similarly, hChr 2 was truncated at *CD8A* locus and a *lox*P sequence was inserted into *WHZ30-4* locus.

In terms of broader needs for functional genomic engineering, it is desirable to develop a general approach to the problem of introducing and stably maintaining hChrs containing defined regions in mice. To meet this challenge, we have developed a chromosome-cloning system that employs a combination of Cre/*lox*P-mediated chromosome translocation with telomere-directed chromosome truncation in homologous recombination-proficient chicken DT40 cells (Kuroiwa *et al.*, 1998, 2000). Using this approach, it is possible to introduce very large (> 1 Mb) and well-defined hChr regions into a stably maintained hChr 14 fragment-based mini-chromosome vector. *Figure 3.4* presents a schematic summary of this unique and novel cloning system.

DT40 cells containing the HAC carrying the 10 Mb-sized Igλ-bearing, which was engineered as described in *Figure 3.4*, could be sorted by FACS because the HAC contains a GFP marker. Repeated FACS sorting and culture under selective conditions was found to lead to significant enrichment of DT40 cells containing the HAC (*Figure 3.5*). The HAC was transferred to ES cells and its mitotic stability was examined. The HAC incorporating a 10 Mb insert of the poorly-retained hChr 22 fragment was maintained at 99.8% retention per cell division throughout 45 day non-selective culture. This level of retention is equivalent to that of the hChr 14-based SC20 vector itself (data not shown). This contrasts sharply with the behaviour of hChr 22 fragment, which was rapidly lost during 15 days in culture under non-selective conditions.

Using ES cells containing this IgH and Igλ-bearing HAC, we have obtained healthy chimeric mice with varying degrees of chimerism (20–80% agouti coat colour). High expression levels of human Ig-heavy and light λ chains were achieved (data not shown) in the sera of these HAC chimeras. On the other hand, because of the poor retention of hChr 22, chimeras created from ES cells containing both the hChr 14 and 22 fragments individually showed much lower expression levels of the human λ chain (data not shown).

Future development of TC technology

TC mouse technology using specifically engineered HACs may enable us to clone and express *in vivo* various defined human chromosome regions that, due to their large size and complexity structure (e.g. dystrophin, unrearranged T cell receptor loci, the MHC cluster, the P450 cluster and multigenic disease regions) have been difficult or impossible to clone by conventional cloning methods. Although the ES cells with germline differentiating potency are currently available only in the mouse, chromosome transgenesis in large farm animals may be feasible using recently developed cloning technologies (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998). For example, it might be possible to generate cows or sheep producing human Igs by nuclear transfer from bovine or ovine fibroblasts containing hChr fragments or genetically engineered HACs with human *Ig* loci. Such animals might provide sources from which pathogen-specific human γ-globulins could be obtained for the treatment of infectious diseases. The HAC technique might also be used in the construction of artificial chromosome vectors for human gene therapy. However, at present, the large size of the artificial chromosome makes transfer a severe problem.

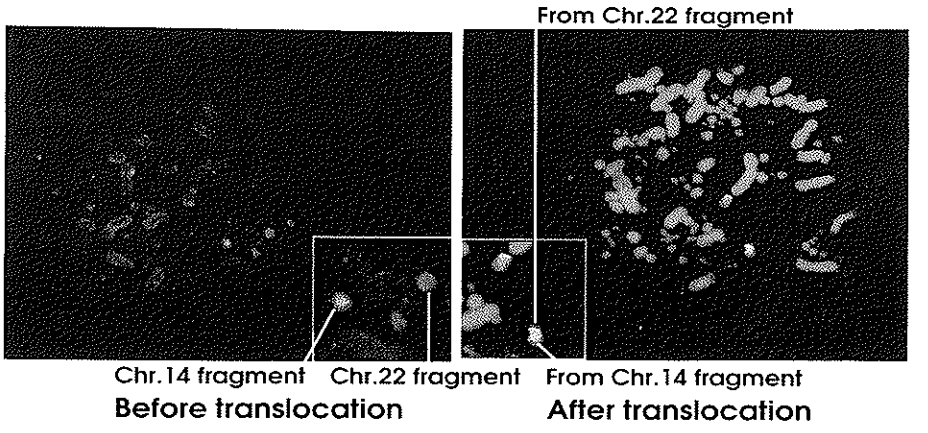

Figure 3.5. Verification of successful isolation of the cells containing λ-HAC generated from translocation between the hChr 14 and 22 fragments by FISH analysis in the FACS-sorted DT40 cells (the right panel) using hChr 14 and hChr 22-specific probes. The left panel shows the FISH in the DT40 cells containing both hChrs before transfection with Cre-expression vector.

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