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Microcell-mediated chromosome transfer (MMCT) of human artificial chromosome (HAC) following cryopreservation for the ready-made use.

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Summary

Microcell-mediated chromosome transfer (MMCT) is a technology which enables to transfer a single and intact mammalian chromosome or its fragment containing some Megabase-sized stretches from donor to recipient cells(Fig1, Table1). Human artificial chromosomes (HACs) for genetic correction or modification have been transferred to various cell types e.g., iPSCs and MSCs by fusing microcells with the recipient cells(Fig 2, 3 & 4, Table 2). Polyethylene glycol (PEG) has conventionally been used for the microcell fusion. However, PEG is not suite to all type of cells as a fusogen, because it has cytotoxicity against some cell types. The colony efficiency of fusion between microcells and recipient cell is about 1 x 10-^{6} ~5 x 10^{-5}. Measles virus fusogen envelop proteins that are hemagglutinin (H) and fusion (F) proteins were expressed on the surface of microcells(Fig5). These proteins can mediate to fuse microcells and recipient cells. Hence, the cytotoxicity was reduced and improved the efficiency of MMCT to 1 x 10^{-4} (Table 3).

The conventional MMCT method has been performed immediately after purification of microcells. The timing of isolation of microcells and preparation of recipient cells are very important. Thus, ready-made microcells can make the MMCT easier. Here, we established a cryopreserved method to store microcells at -80 degree(Fig6). We compared the conventional and the cryopreserved methods for the efficiency of MMCT and the stability of human artificial chromosome (HAC) when transferring to human HT1080 cells. Drug-resistant cells appeared after selection in culture with the tagged selection marker gene, Blasticidin on the HAC(Fig7). The chromosome transfer efficiency was determined by counting the total number of stable clones expressing EGFP obtained in each experiment. The presence of the HAC in microcell hybrids was confirmed by FISH analyses(Fig8). There is not a significant difference between the two methods for the chromosome transfer efficiency and retention rate of HAC.

Thus, the cryopreserved method with the MV-H and F proteins as a fusogen is an improved simple MMCT protocol(Fig9).

Fig.1 Construction of human monochromosomal hybrids via microcell-mediated chromosome transfer

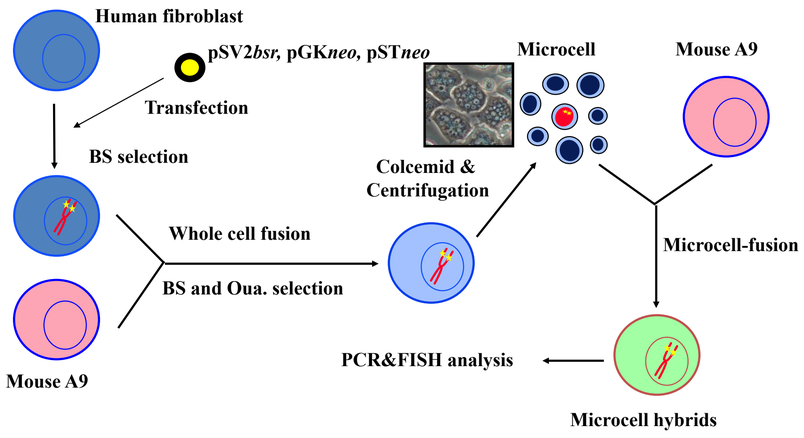
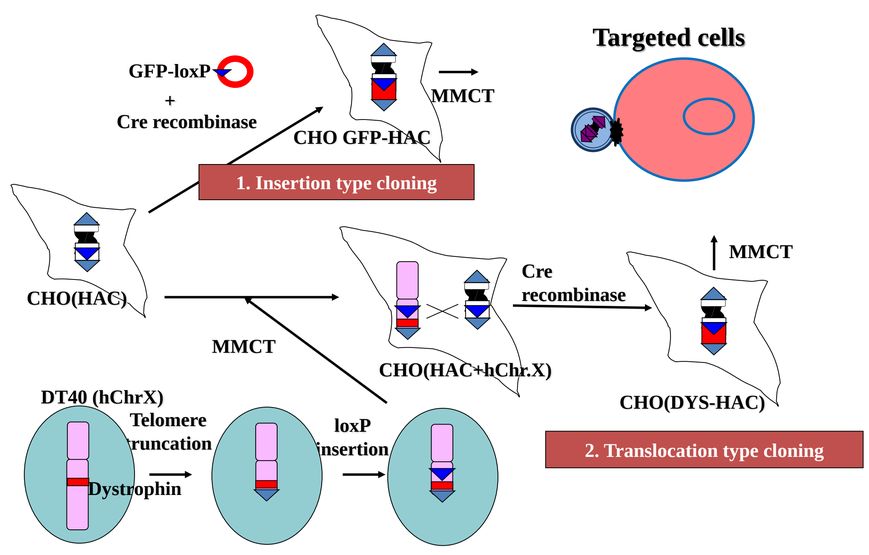


Fig.2 Gene cloning system on the HAC vector

HAC can be transferred to other cells by microcell-mediated chromosome transfer (MMCT).



Human artificial chromosomes for gene delivery and the development of animal models. Kazuki Y. and Oshimura M., Mol Ther 2011. doi: 10.1038/mt.2011.136.

Table.1 Application of chromosome transfer and engineering

1. Mapping and isolation of genes responsible for genetic disorders.
2. Mapping and isolation of tumor suppressor genes and senescence genes.
3. Mapping and isolation of imprinted genes and the mechanisms.
4. Humanized mouse models (human antibodies, human P450 mouse).
5. Trisomy models (Down syndrome model mouse, trisomy cell and its consequence)
6. Human artificial chromosome (Table 2)

- gene/cell-therapy
- gene function and interaction
- Protein production
- Monitoring system (differentiation, function, toxicity and function)
- Production of iPS Cells

Table 2. Examples of genes delivered by our human artificial chromosome *via* MMCT

Loaded genes	DNA type	Insertion method	Recipient cells or animals	Aims	References
Human IgH and Igk/Igλ	Genomic	Cre/loxP (translocation type)	mouse, caw	Production of humanized antibody	Kuroiwa et al., 2000, 2002, 2009
Human CYP3A cluster	Genomic	Cre/loxP (translocation type)	mouse	Prediction of human drug metabolism and toxicity	Y. Kazuki et al., unpublished results.
Ubc-hTERT-IRES-GFP	cDNA	Cre/loxP	HFL-1	Life-span extension of normal fibroblast	Shitara et al., 2008
PGK-ScFv-gp130-IRES-EGFP	cDNA	Cre/loxP	7TD1, hBM MNC	Antigen-mediated growth control	Yamada et al., 2006 Kawahara et al., 2007
TR-DNA-PKcs	cDNA	Cre/loxP	V3	Tetracycline-mediated inducible gene expression system	Otsuki et al., 2005
Mouse CD40L	Genomic	Cre/loxP	Jurkat, U937	BAC-PAC-mediated gene expression system for gene therapy	Yamada et al., 2008
Human HPRT	Genomic	Cre/loxP	CHO hprt−/−, HeLa hprt−/−	TAR cloning-mediated or ready made PAC-mediated gene insertion	Ayabe et al., 2005 Kazuki et al., 2008
HSP70-Insulin	cDNA	Cre/loxP	HT1080	Heat-regulated gene expression system	Suda et al., 2006
Human TP53	Genomic	Cre/loxP	mGS p53−/−, mouse	Genetic correction in mGS cells	Kazuki et al., 2008
OPN-EGFP	cDNA	Cre/loxP	hiMSC	Lineage-specific gene expression	Ren et al., 2005
CMV-human EPO	cDNA	Cre/loxP	HFL-1	Therapeutic protein expression in normal fibroblast	Kakeda et al., 2005
UBC-human EPO	cDNA	Cre/loxP	CHO, h primary fibroblasts	Production of high efficiency human protein.	Kakeda et al., 2011
OC-GFP	cDNA	Cre/loxP	CHO	Evaluation system for bioactive substances	Takahashi et al., 2010
MC1-HSV-TK	cDNA	Homologous recombination	hiMSC	Suicide gene- and MSC-mediated treatment of glioma	Kinoshita et al., 2010
NBS1 and VHL	Genomic	Cre/loxP	GM07166 and RCC 786-0 (Deficient cell lines)	Genetic correction of NBS1 and VHL	Kim HJ et al., 2011
Human dystrophin	Genomic	Cre/loxP (translocation type)	hiMSC, mouse, mdx-iPS, DMD-iPS	Genetic correction of DMD in iPS cells	Hoshiya et al., 2009 Kazuki et al., 2010 Tedesco FS et al., 2011
Yamanaka factors and p53shRNA	cDNA	Cre/loxP	MEF, mouse iPS	Generation of iPS cells	M. Hiratsuka et al., 2011 Kakeda et al., 2011
CAG-human FVIII (1–16 copies)	cDNA	Cre/loxP	CHO hprt−/−, hiMSC	Copy number-dependent gene expression system	H. Kurosaki et al., 2011

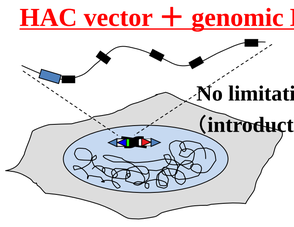
Human artificial chromosomes for gene delivery and the development of animal models. Kazuki Y. and Oshimura M., Mol Ther 2011. doi: 10.1038/mt.2011.136.

Fig.3 Characteristics of our human artificial chromosome (HAC) vector

plasmid	cosmid	BAC	YAC	Chromosome	
1 kb	10 kb	100 kb	1 Mb	10 Mb	100 Mb

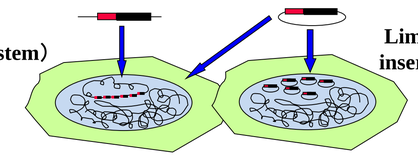
HAC vector

HAC vector ＋ genomic DNA



- No integration in genomic DNA
- Arbitrary copies and stable
- Physiological regulation
- No over-expression/no silencing

virus／expression vector
exogenous promoter ＋cDNA

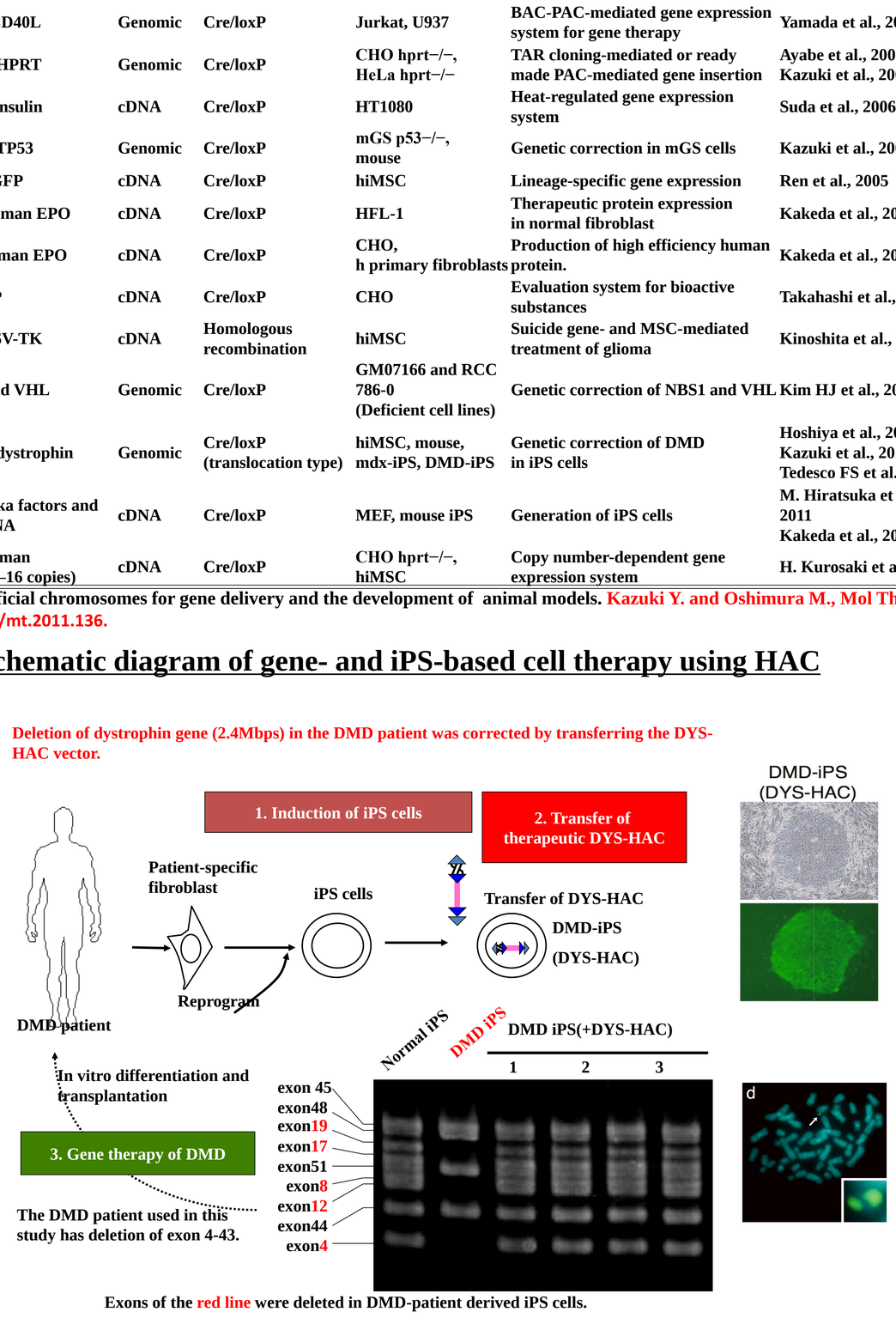


- Genomic disruption
- Copy number is unpredictable
- Gene regulation by exogenous promoter
- Overexpression or silencing

Human artificial chromosomes for gene delivery and the development of animal models. Kazuki Y. and Oshimura M., Mol Ther 2011. doi: 10.1038/mt.2011.136.

Fig.4 Schematic diagram of gene- and iPS-based cell therapy using HAC

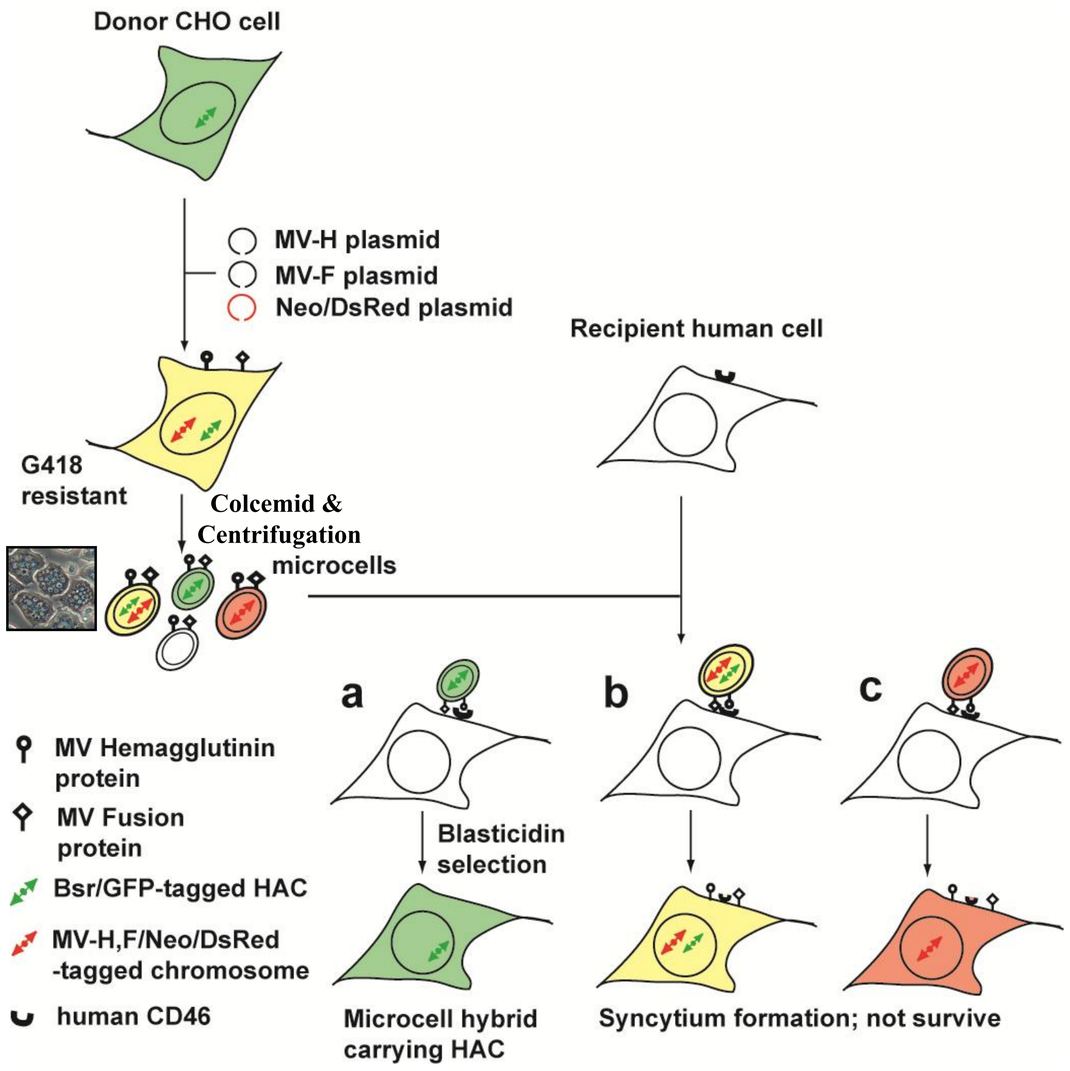
Deletion of dystrophin gene (2.4Mbps) in the DMD patient was corrected by transferring the DYS-HAC vector.



Exons of the red line were deleted in DMD-patient derived iPS cells.

Complete genetic correction of iPS cells from Duchenne muscular dystrophy. *Kazuki et al, 2010 Mol Ther.* **doi:10.1038/mt.2009.274**

Fig.5 Improvement of microcell-mediated chromosome transfer using measles virus fusogenic envelope proteins with a surface receptor on human cells



Rationale for microcell fusion using an MV fusogen.
Donor CHO cells carry a human artificial chromosome (HAC) tagged with blastcidin-resistant (Bsr) and GFP gene. The CHO cells are transfected with plasmids encoding the MV-Fusion (MV-F) and Hemagglutinin (H) proteins and selection marker (Neo/DsRed). Microcells are prepared from the G418-resistant CHO donors and gave to recipient human cells. They are commonly coated with MV fusogen but contain different chromosomes. The donor-derived chromosome within the microcell is donated to the recipient cells by microcell fusion, which is mediated by interaction of the MV fusogen and the CD46 receptor presented on the surface of recipient cells. (a) The bsr-tagged HAC is rescued in mycrocell-hybrid by selection culture with Blasticidin. (b, c) On the other hand, introduction of the MV-H/F-tagged chromosome into recipient cells results in de novo synthesis of H/F proteins, leading to cell death caused by syncytium formation with the surrounding cells.

Exploitation of the interaction of measles virus fusogenic envelope proteins with the surface receptor CD46 on human cells for microcell-mediated chromosome transfer. Katoh *et al. BMC Biotechnology* 2010, doi:10.1186/1472-6750-10-37

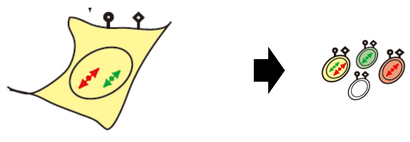
Table 3. Yield of drug-resistant microcell hybrids by using MV fusogen and PEG

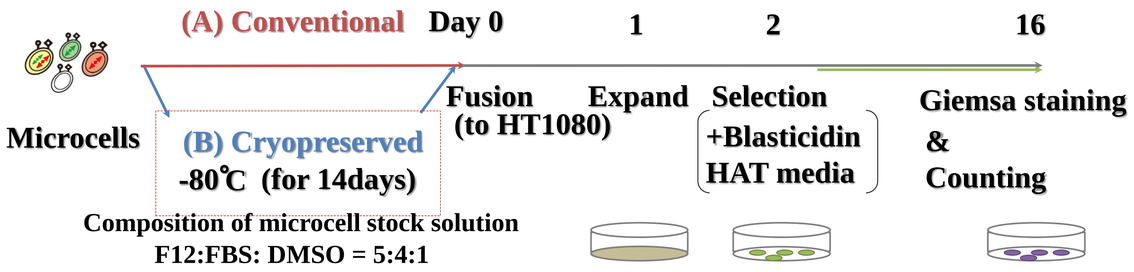
	Colony number1	
Amount of applied microcells	MV -fusion	PEG-fusion
2×10^5	51	5
4×10^5	86	6
8×10^5	94	15
1×10^6	75	13
2×10^6	60	22

1. Number of Blasticidin-resistant colonies obtained from 2×10^6 of HT1080 cells.

Fig.6 Microcell-mediated chromosome transfer of human artificial chromosome following cryopreservation for the ready-made use.

CHO donor cells have expressed the MV-H and F protein.



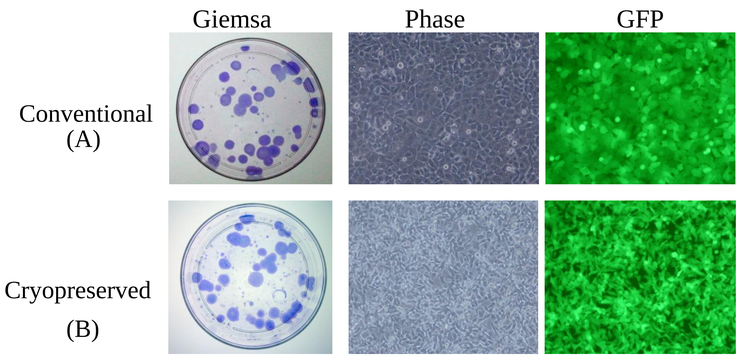


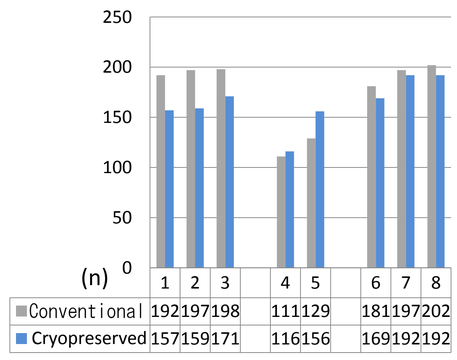
The protocol;

CHO donor cells harboring a HAC were seeded 6.0x10^{5}cells donor per flask. And, thirty-six flasks were prepared. These donor cells were treated for 72 hours at a concentration of colcemid 0.05μg/mL, which made microcells. Then, microcells were collected by centrifuge 8,000 rpm for 1 hour. The obtained microcells were dispensed in six tubes, three tubes were immediately used for cell fusion experiments as a conventional method (A). The remained three tubes cryopreserved in -80 degrees for 14days using a general cell freezing method as cryopreserved method (B). The composition of this microcell stock solution was a ratio of F12:FBS: DMSO = 5:4:1. After that, redissolved microcells were used for cell fusion experiments. On the other hand, HT1080 as recipient cells were prepared 2x10^{6}cells per cell fusion experiments.

After 24 hours, these fused microcell hybrids were expanded to 6 of 10cm dishes. After a further 24 hours, blasticidin S was added to a concentration of 8μg/mL and 1x HAT media.
GFP-positive and resistant colonies were obtained after 14 days and counted by Giemsa staining.

Fig.7 Introduction of human artificial chromosome by the cryopreserved method.



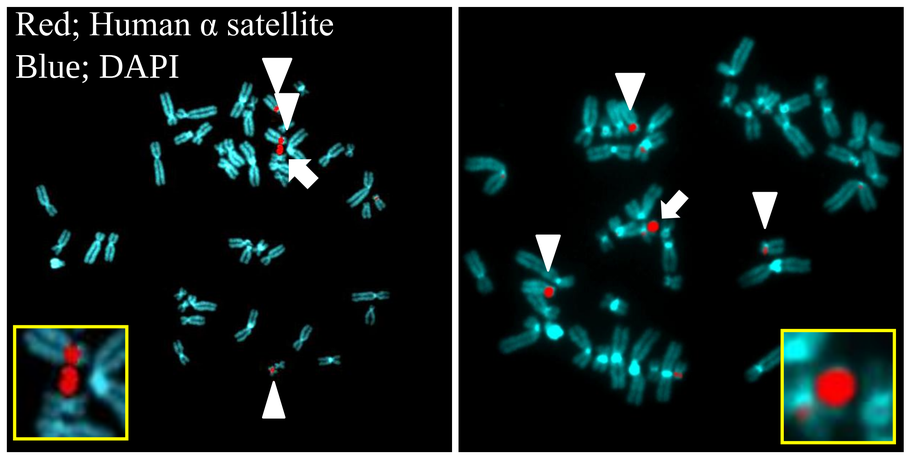


When compared with the conventional method, the cryopreserved method has no significant difference in drug-resistant colony efficiency.

The left representations show that the GFP- positive rate of the obtained microcell hybrids by microscopic observation.
The right bar graph shows the number of drug-resistant colonies were obtained from each microcell fusion experiment. (n) means the experimental #. (1, 2, 3), (4, 5), (6, 7, 8) used the same lot of the obtained microcell.

Fig.8 HAC retention rate by FISH analysis

A B

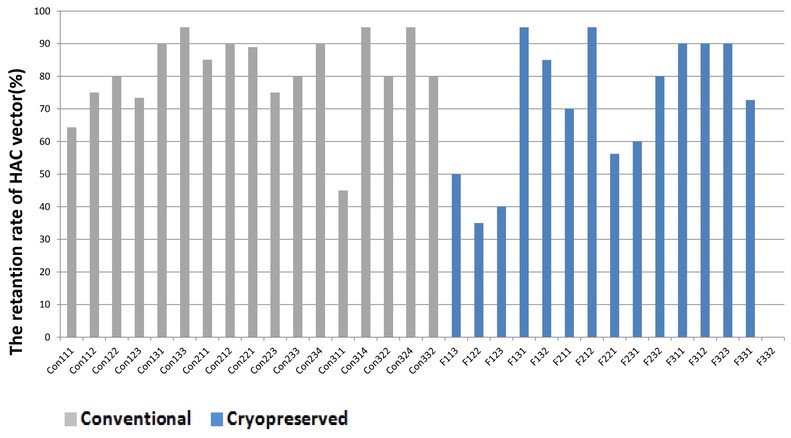


Cryoreserved **Conventional** **Parental cell**

Even when using either method, HACs had been maintained in HT1080 cells.

Allows show HACs introduced in HT1080 cells. Allow heads shows alphoid sequences on human chromosome #13or 21.
HACs are enlarged in each window.

The retention rate of HAC vectors



This graph shows the retention of HAC in each obtained drug-resistant clone.

When compared with the conventional method, the cryopreserved method has no significant difference in the retention rate of HAC vectors.

Conclusions

- **The cryopreserved method with the MV-H and F proteins as a fusogen is an improved simple MMCT protocol.**
- **When microcells cryopreserved, the cryopreserved method could obtain similar number of drug-resistant colonies compared with the conventional method.**
- **There were no significant difference in the retention rate of HACs in HT1080 between each method.**

This method can be practiced by everyone, and does not require technical skills and specialized facilities and equipments.
HACs are available in the table 2 as ready-made vectors, on your requests.

Fig.9 New conceptual diagram of MMCT