Requisiti di un vettore ideale

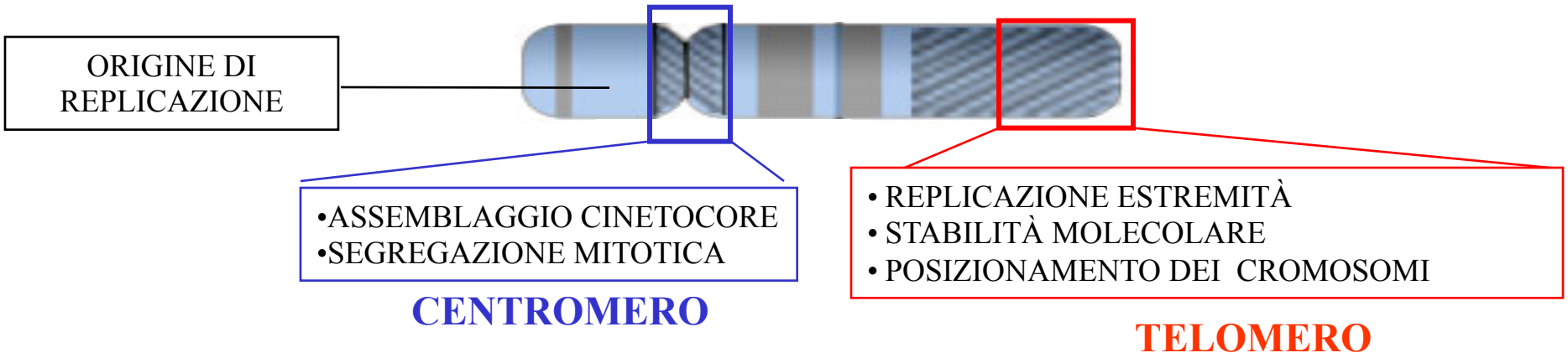
- **minima invasività**
- **selettività del bersaglio**
- **assenza di immunogenicità**
- **elevata capacità di clonaggio**
- **stabilità nel tempo**
- **corretta ploidia**
- **manipolabilità**
- **dimensioni ridotte**

MAC (Mammalian Artificial Chromosome)

- **è una molecola che mima il comportamento di un cromosoma naturale**

- **è costituita dagli elementi strutturali dei cromosomi naturali**

ELEMENTI STRUTTURALI



PROPRIETÀ

- **BASSO NUMERO DI COPIE**
- **ELEVATA STABILITÀ**
- **ELEVATA CAPACITÀ DI CLONAGGIO**
- **PICCOLE DIMENSIONI (1-5% DEI CROMOSOMI NATURALI)**
- **ASSENZA DI REAZIONI IMMUNITARIE (possibile reazione da transgene)**

centromere function

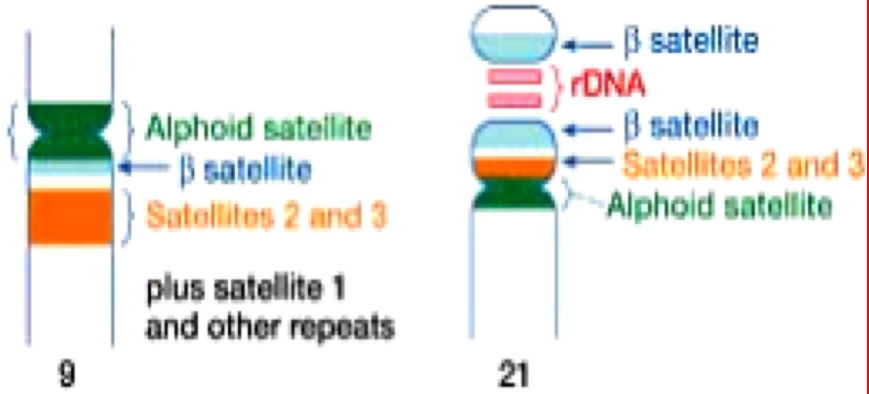
the centromere is the chromosomal location for the assembly of the kinetochore, which provides sites for microtubules attachment in chromosome segregation during cell division.

it is epigenetically inherited as a region marked by the Cenp-A nucleosomes.

Cenp-A has been identified as a histon H3 variant

centromeric DNA

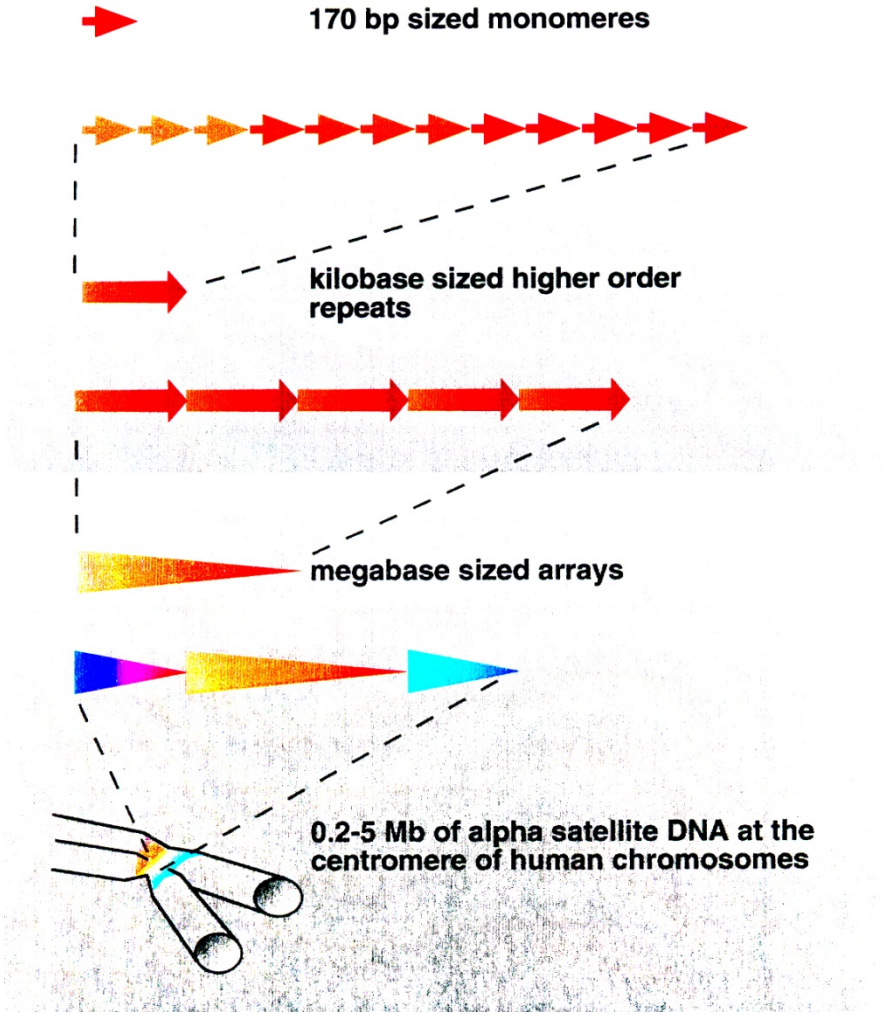
- satellite DNA
  - satellite 1
  - satellite 2
  - satellite 3
- satellite alfa
- satellite beta
- satellite gamma
- satellite 42-bp
- satellite Sn5



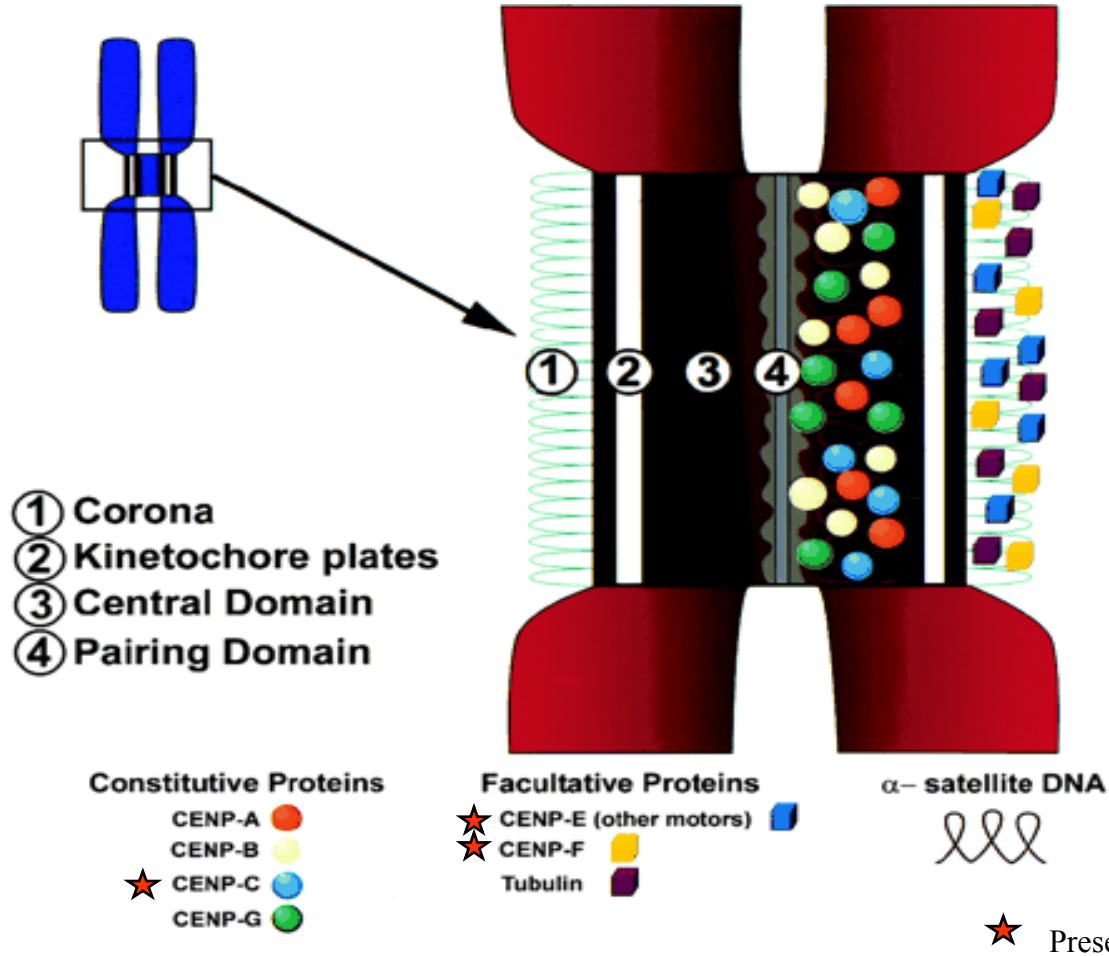
alphoid DNA families

Famiglie sopracromosomali	Cromosomi	Tipo di monomeri	Tipo di organizzazione dei monomeri
1	1, 3, 5 ,6 ,7,10, 12, 16, 19	tipo J	… - J1 - J2 - . . .
2	2, 4, 8, 9, 13, 14, 15, 18, 20, 21, 22	tipo D	. . . - D1 - D2 - . . .
3	1, 11, 17, X	tipo W	. . . - W1-W2-W3-W4-W5-.
4	13, 14, 15, 21, 22, Y	tipo M	. . . - M1 - . . .
5	5, 7, 13, 14, 19, 21	tipo R	. . . - R1 - R2 - . . .

structural organization of the alphoid DNA

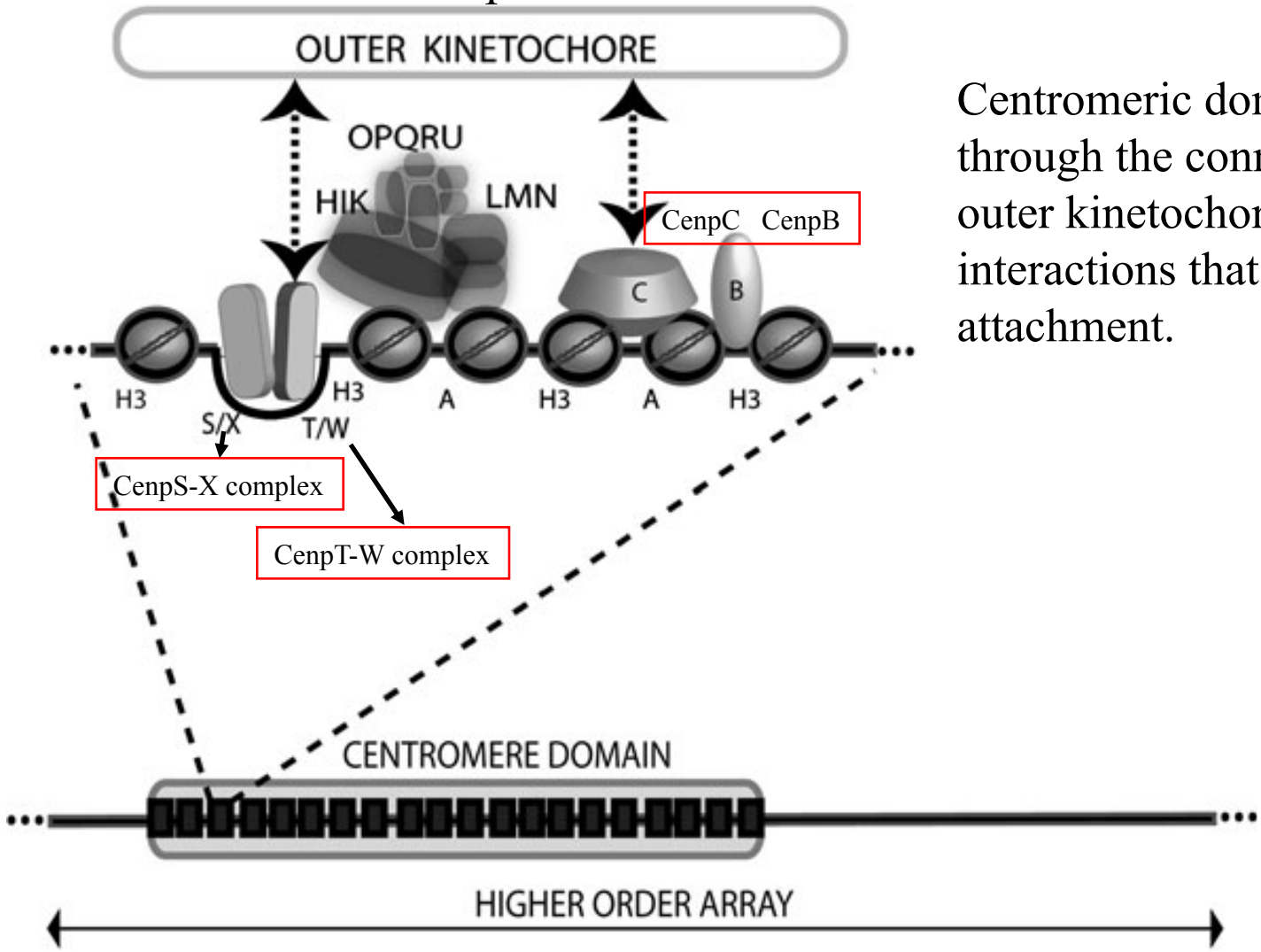


the different component of mammalian centromeres



- **CenpA, centromere-specific H3 variant, it is essential for centromeric chromatin**
- **CenpC, it is associated with centromere activity, absent in inactive centromere**
- **CenpB, it is not required when the centromeric chromatin is already assembled but it is essential for de novo centromere formation**

Kinetochore protein interactions with centromeric DNA

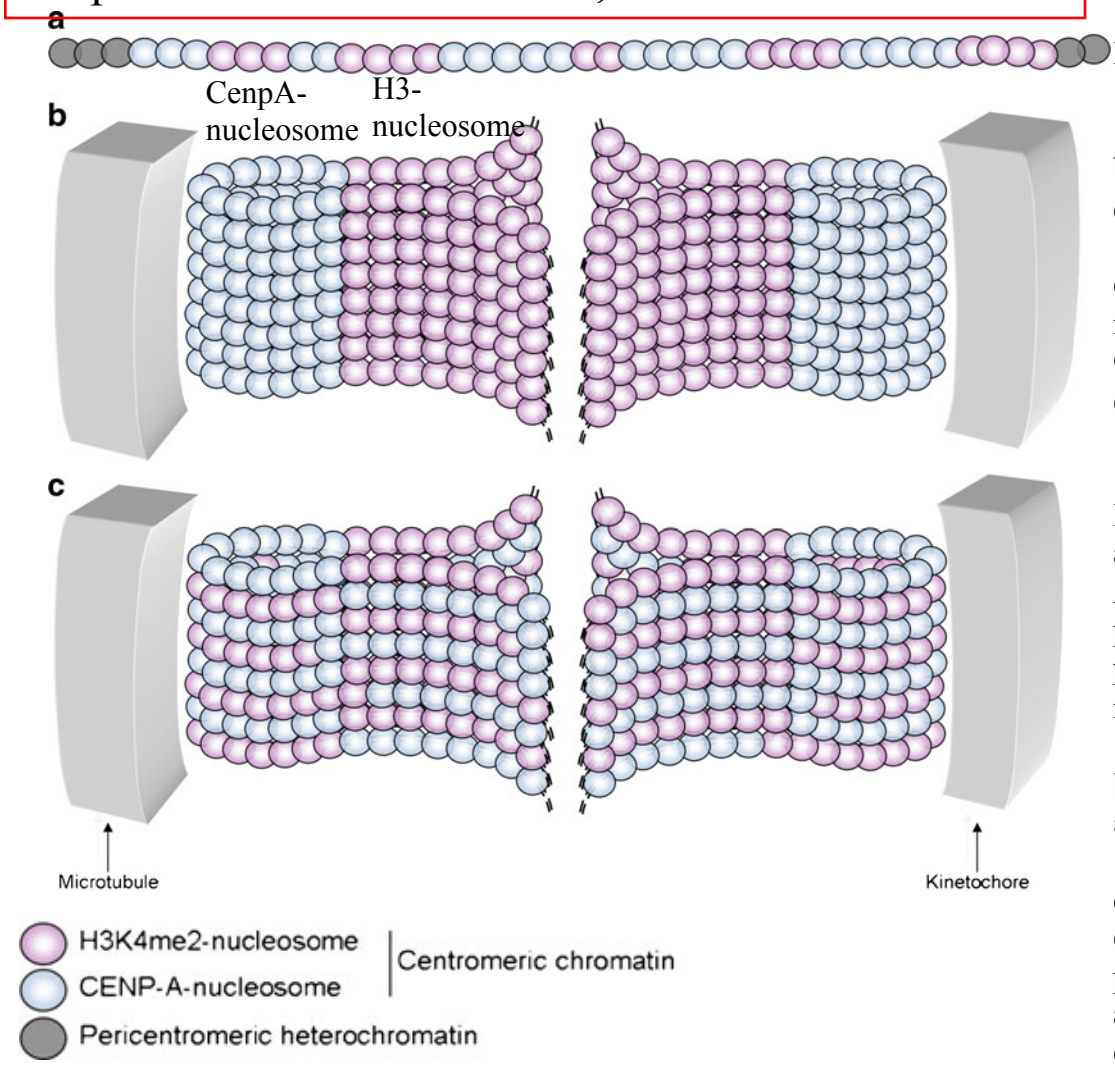


Centromeric domains are organized through the connection of inner and outer kinetochore protein interactions that facilitate spindle attachment.

A model for kinetochore proteins is shown here with a focus on their interaction with the underlying DNA, and centromeric chromatin (CENP-A noted as A, nucleosomes containing histone H3 as H3, and centromere proteins that are shown to directly interact with the underlying DNA: CENP-B (B), CENP-C (C), CENP-T-W complex (T/W), and CENP-S-Xcomplex (S/X)). The protein-determined connection to the outer kinetochore is indicated with black arrows. Additional protein complexes that characterize the constitutive centromere- associated network (CCAN) are indicated in gray.

Models of spatial organization of centromeres during chromosome segregation

cenpA substitutes H3 in some, but not all nucleosome



a Schematic of the CENP-A and H3 (dimethylated on lysine 4—H3K4me2) alternating domains at centromeres

b Model of CENP-A domain exposure at the surface of the mitotic centromere.
A recent advance comes from three-dimensional views of mitotic chromosomes obtained by electron microscopy (EM), which revealed a precise arrangement of centromeric fibers, so that **CENP-A domains are extruded to the surface of the constriction, whereas H3 domains are buried deeper inside**

c Model of boustrophedon organization of the mitotic centromere. However, recent data derived from super-resolution microscopy and field emission scanning EM have challenged this model. According to the alternative model, chromatin folding leads to planar sinusoidal layers of centromeric fibers, forming a boustrophedon, which results in the exposure of both, CENP-A and H3 nucleosomes, at the surface of the constriction.

boustrophedon, from right to left and from left to right in alternate lines.
Una **scrittura bustrofedica** è una scrittura che non ha una direzione "fissa" ma procede in un senso fino al margine scrittorio e prosegue poi a ritroso nel senso opposto, secondo un procedimento "a nastro", senza "andate a capo" ma con un andamento che ricorda quello de solchi tracciati dall'aratro in un campo. L'etimologia della parola ricorda infatti l'andamento di un bue durante l'aratura (dal greco βοῦς, "bue", e στρέφειν, "girare, invertire").

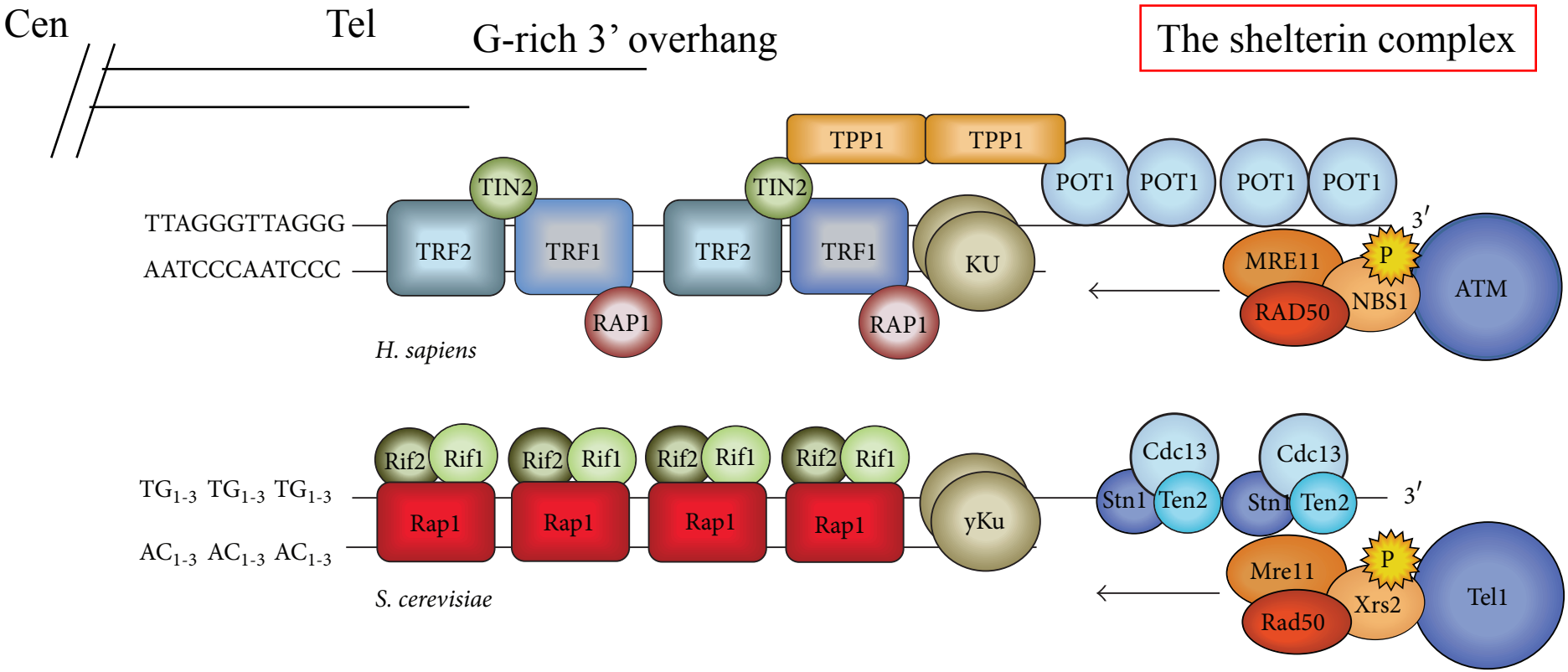
Telomeres function

- **ends protection from recombination**
- **allow complete replication of the chromosomal ends**
- **direct chromatin organization**
- **cooperate to chromosome segregation during mitosis and meiosis**

Telomere length and sequence

Protozoi *(Oxythricha, Tetrahymena)*	20-30 bp	$(C_4A_4)n$ or $C_4A_2)n$
Yeast *S. cerevisiae*	350-500 bp	$(C_{1\text{-}3}A)n$
topo *mus musculus*	60-100 kb	$(T_2AG_3)n$
uomo	3-20 kb	$(T_2AG_3)n$

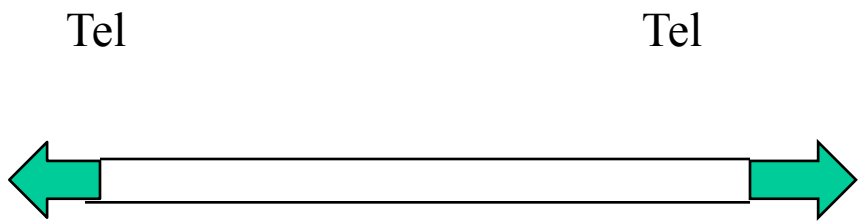
Telomere structure in human and *S. cerevisiae*.



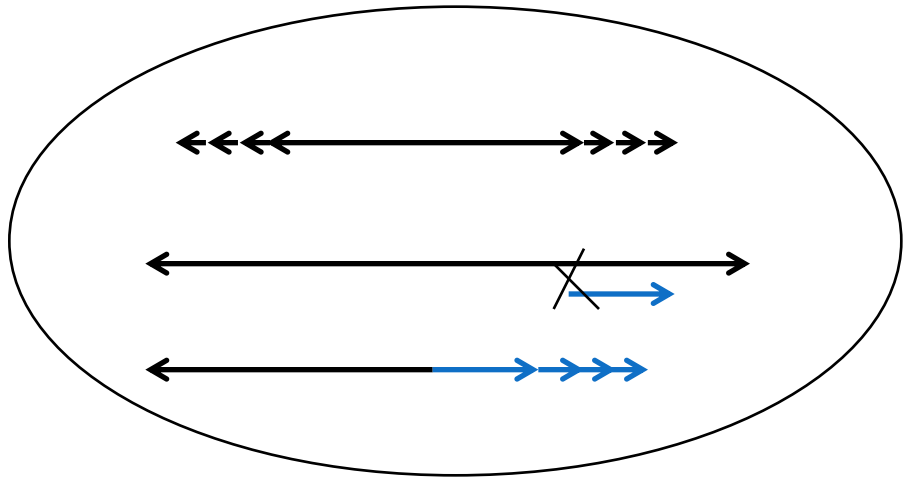
Human telomeres consist of kilobases of TTAGGG repeats, ending with a) 3' overhang, G-rich strand. The shelterin complex includes six proteins: TRF1 and TRF2, which bind directly the double-stranded telomeric DNA and are held together by TIN2, RAP that interacts with TRF1, POT that associates with telomeric ssDNA, and TPP. These factors mediate the generation of higher-order structure at chromosome ends (T-loop) by invasion of the single-stranded G-overhang into the double-stranded TTAGGG repeats. In Budding yeast, the double-stranded telomeric sequence is bound by Rap, which regulates telomere length together with Rif1 and Rif2. Cdc13, Ten2 and Stn1 bind to the single strand overhang. In both human and S. cerevisiae, the heterodimeric Ku complex (Ku70/80) interacts with the terminal part of the telomere, providing a protective role. The heterotrimeric complex MRX/MRN (MRE/Mre11, RAD50/Rad50, and NBS1/Xrs2) promotes ATM/Tel recruitment, with a central role in telomere capping and length regulation.

Di Domenico et al 2014. Multifunctional Role of ATM/Tel1 Kinase in Genome Stability: From the DNA Damage Response to Telomere Maintenance

in vivo telomere formation



linear fragments terminating with telomeres are recognized by telomerase which in turn add telomeric repeats.



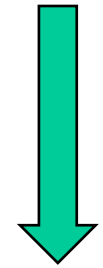
telomerase-mediated telomere lengthening

ori (origin of replication)

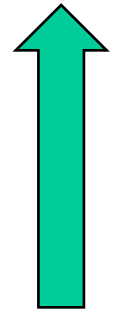
It is claimed that at least one ***ori*** is present every 50 kb. Thus this element is not specifically added but it is supported by whatever DNA fragments larger that 50 kb

COSTRUZIONE DI VETTORI CROMOSOMALI

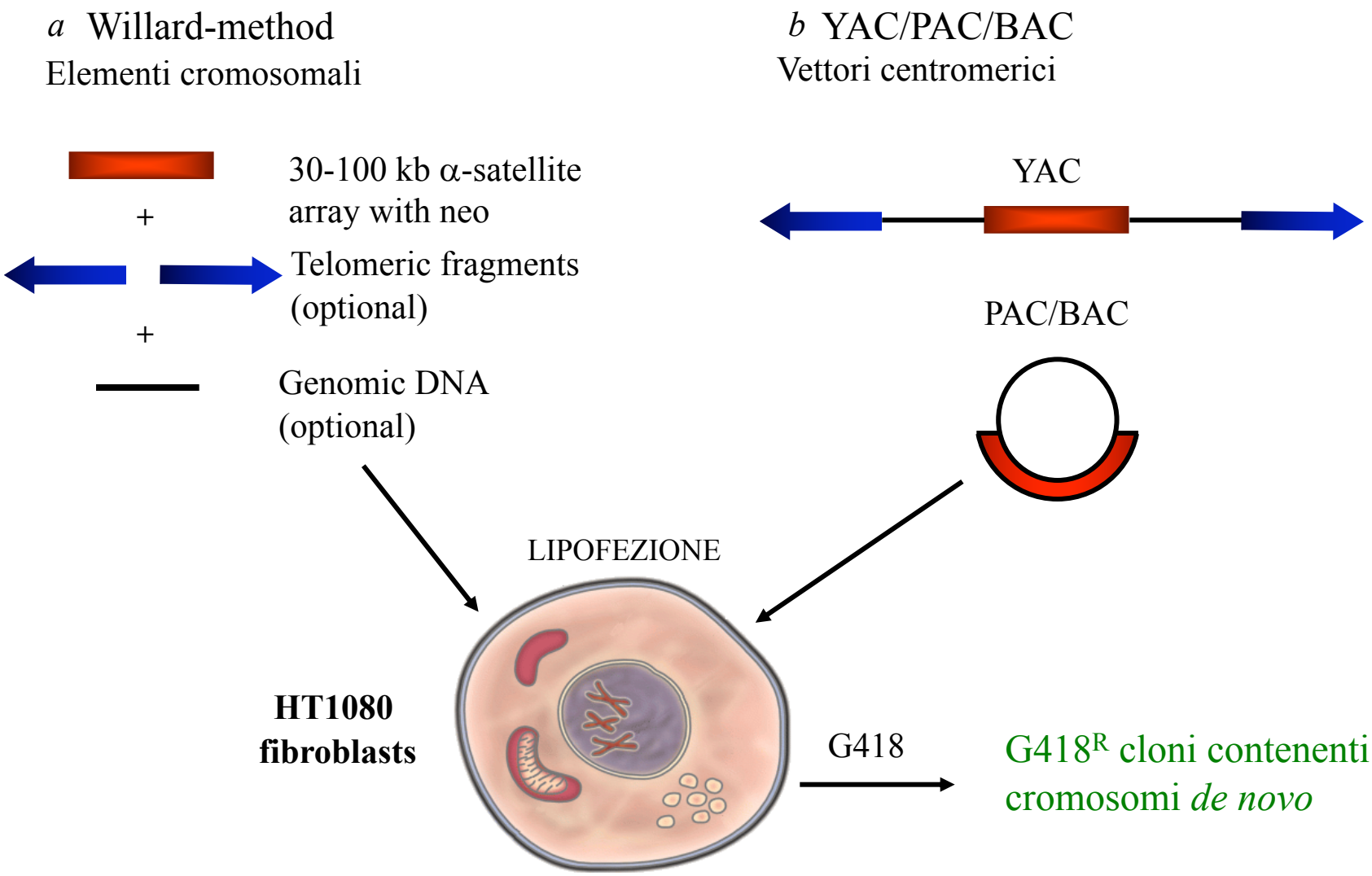
- **Approccio "bottom up" (o assemblaggio *de novo*)**
  - **tecnica di Willard**
  - **tecnologia YAC/BAC/PAC**



- **Approccio "top down"**
  - **frammentazione telomerica**
  - **irraggiamento con raggi γ**



Assemblaggio de novo (bottom up approach)



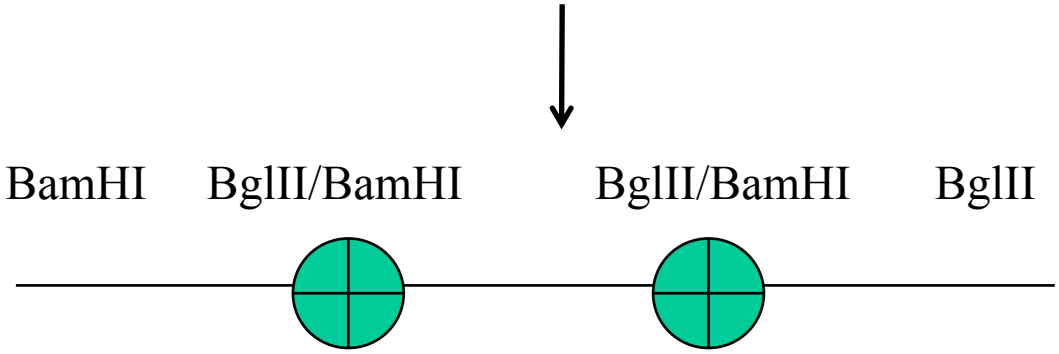
the absolutely required element is:
centromeric DNA

DNA CENTROMERICO SINTETICO
OR
DNA CENTROMERICO CLONATO NEL VETTORE BAC O YAC

DNA centromerico: compenente indispensabile per la costruzione botton up

assemblaggio i*n vitro*

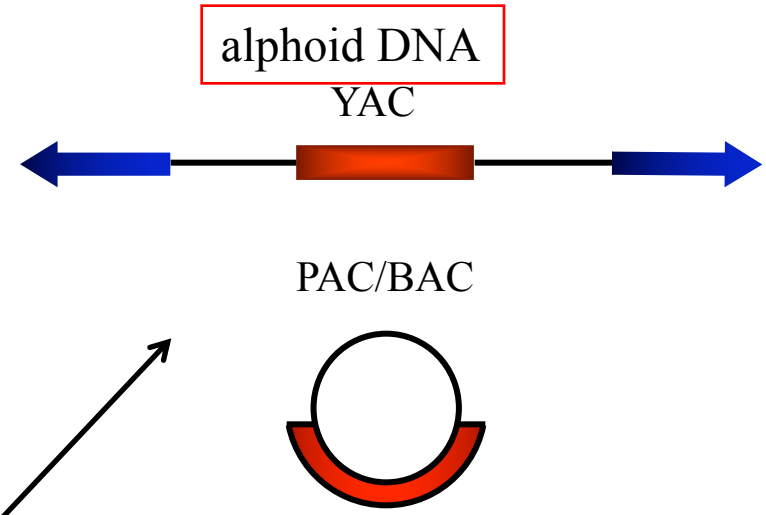
*Bam*HI alphoid DNA *Bgl*II



la digestione con BamHI e BglII lascia un frammento con estremità BamHI e/o BglII e siti interni ibridi BamHi/BglII

la miscela di ligasi viene usata direttamente nel mix di DNA da trasfettare nelle cellule HT1080
alternativamente
clonaggio in un vettore BAC

utilizzo di vettori YAC o BAC



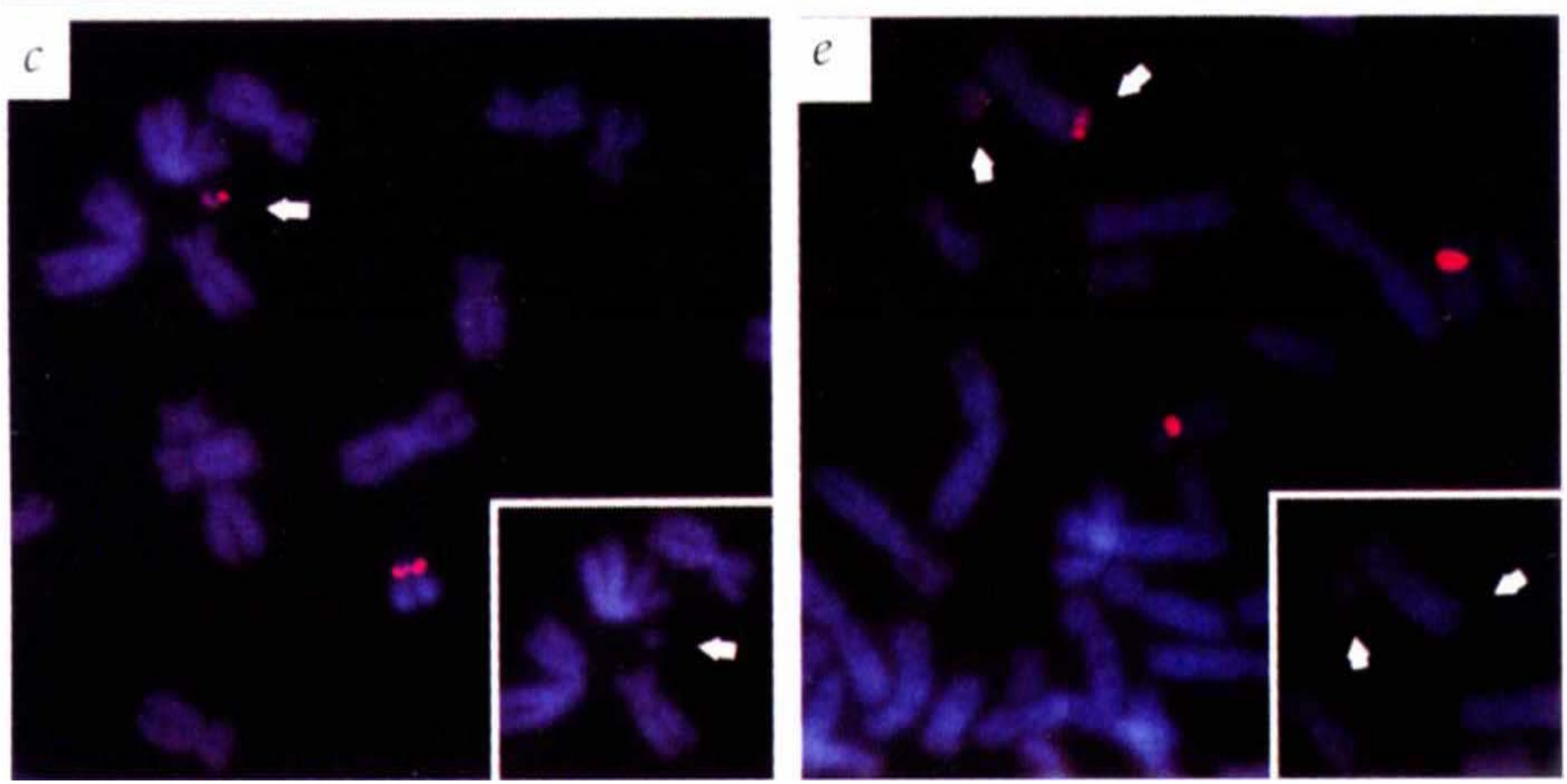
de novo MAC structural features

Mediante analisi FISH (fluorescence in situ hybridization) Si domostrò la presenza di elementi extracromosomiali con le seguenti caratteristiche:

- piccole dimensioni
- contenetnti esclusivamente DNA esogeno (ovvero introdotto nella cellula tramite trasfezione)
- Assenza di DNA endogeno (ovvero appartenente alla cellula ospite)

La formazione dei MAC era strettamente legata alla presenza di DNA centromerico *alpha*
Non tutti i DNA alpha era competenti per la fromazione dei MAC:
alpha 17 ed alpha 21-I inducono la formazione di cromosomi *de novo*;
alpha 21-II non induce la formazione di cromosomi *de novo*

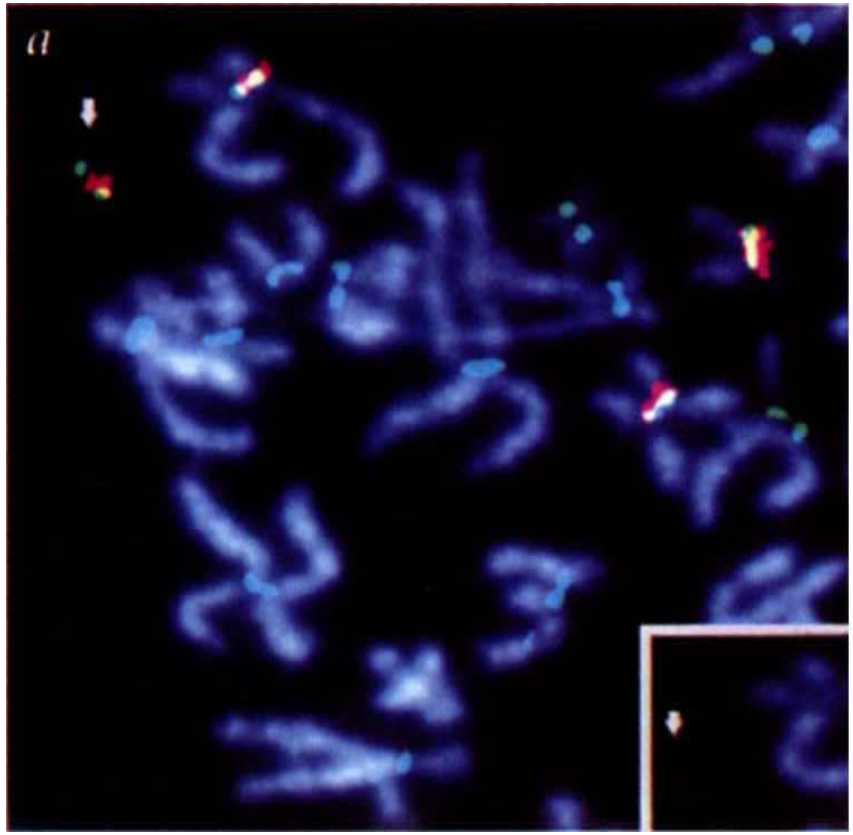
de novo minichromosomes produced by the 17alphoid DNA



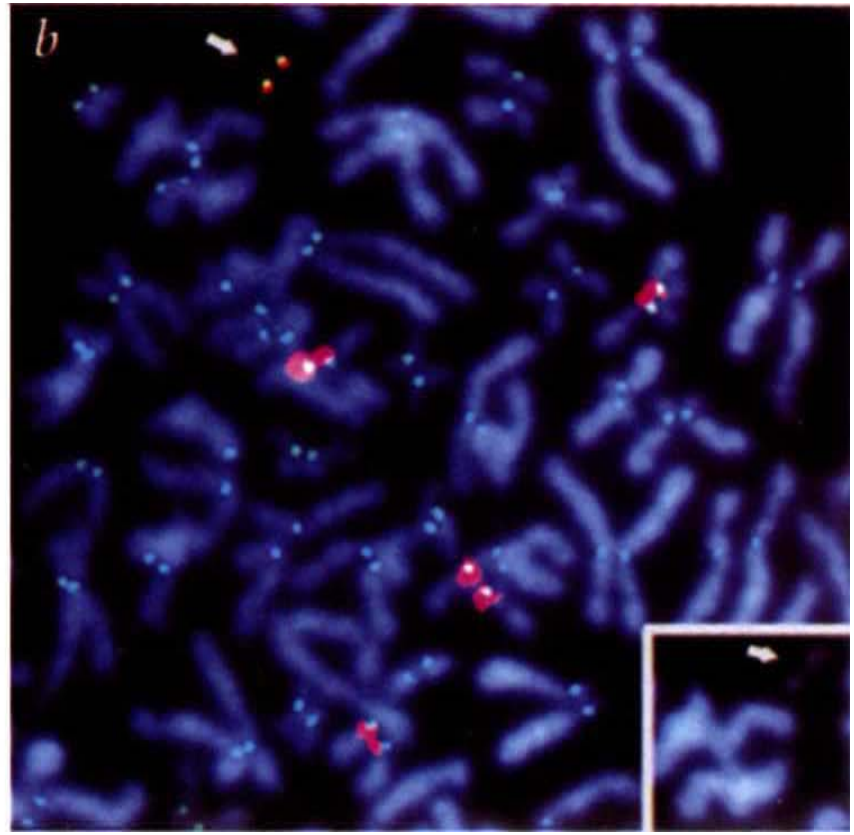
FISH analysis with the 17alphoid probe

centromeric proteins bind the minichromosmes

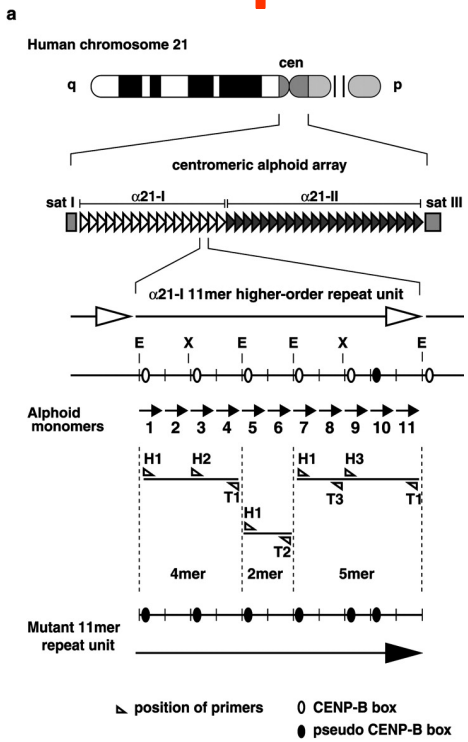
anti CenpC (FITC)+α17(red)

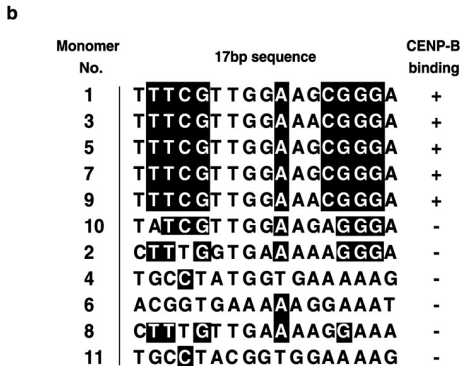


antiCenpE+α17(red)



de novo Chromosome Formation Requires alphoid DNA with functional CENP-B box





a21-I supports MAC formation
a21-II does not support MAC formation

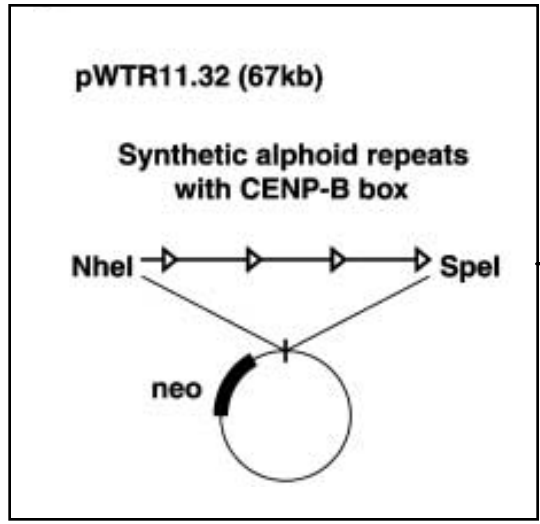
synthetic alphoid with mutated CenB box does not support MAC formation

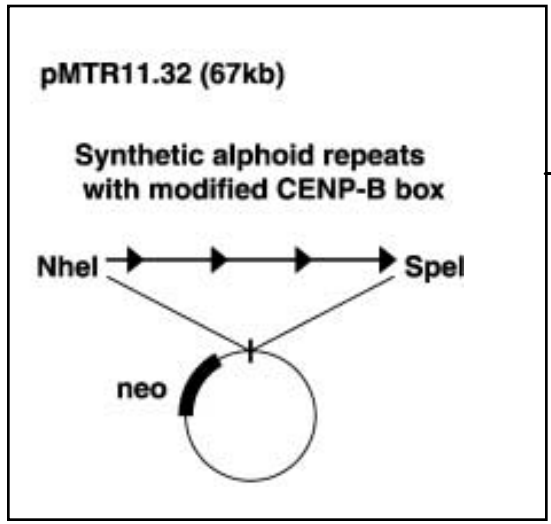
non-alphoid arrays with the CenpB box fail to support MAC formation.

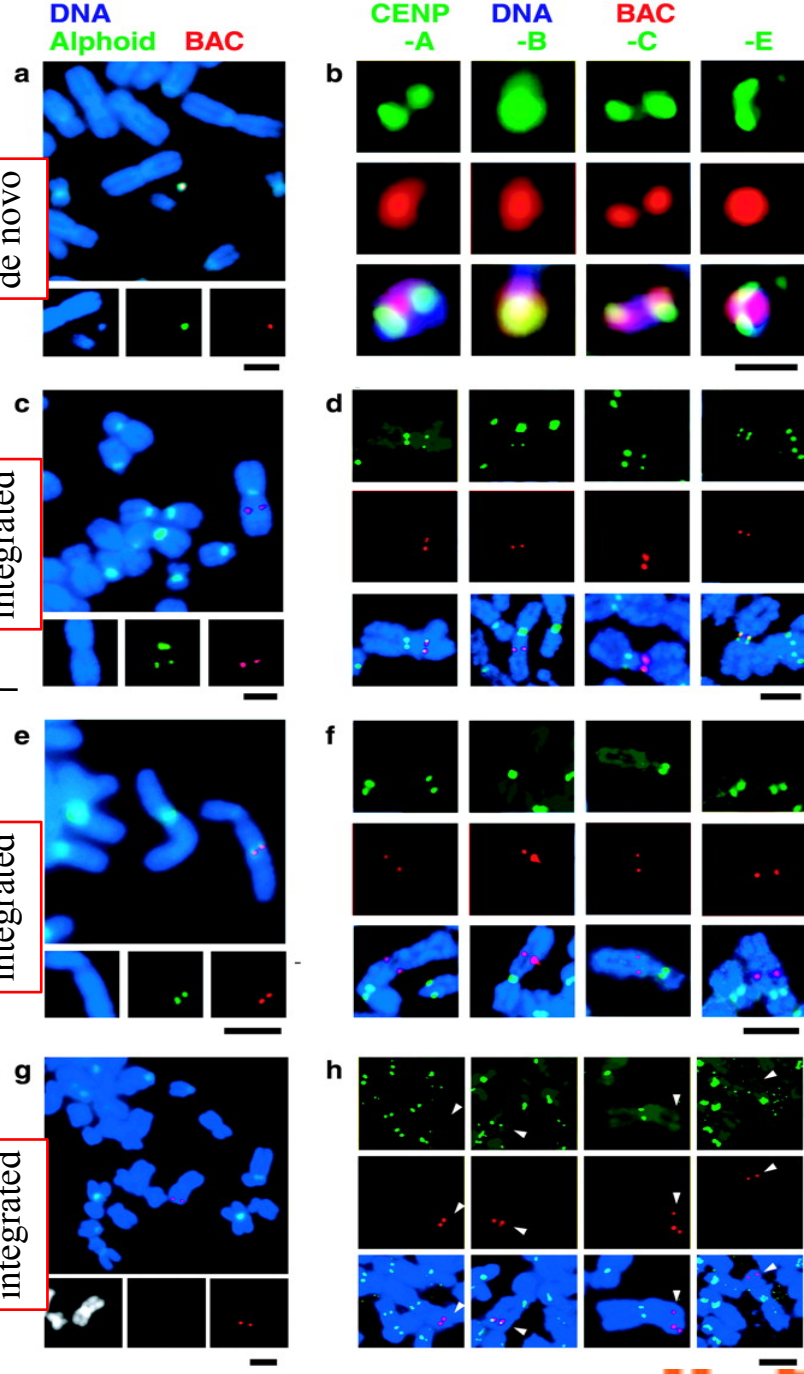
Thus, the interaction between CENP-B and the CENP-B box not sufficient for *de novo* centromere chromatin assembly on the nonalphoid GC-rich sequence, although it is required for centromere chromatin assembly on alphoid DNA.

Ohzeki et al., 2002, JCB 159, 765-775

Examples of extrachromosomal events and centromere protein assembly on introduced DNAs. In all panels, DNA was counterstained with DAPI (blue), and introduced BAC DNA was detected by FISH with a fluorescence-labeled BAC probe (red).







Ohzeki J et al. J Cell Biol 2002;159:765-775

CenpB and alphoid DNA are required for de novo chromosome formation

Table I. **Chromosomal events in the stable cell lines with synthetic 11mer repeats**

Synthetic repeats inserted in BAC (Input DNA)	Experiments	Analyzed cell lines	Chromosomal events of introduced DNA[a]	
			De novo artificial chromosome	Integration into host chromosome (centromeric heterochromatin/arm)
Alphoid repeats with functional CENP-B boxes (60 kb)	1st	15	7	8 (7/1)
(pWTR11.32)	2nd	12	5	7 (6/1)
Alphoid repeats with modified CENP-B boxes (60 kb)	1st	20	0	20 (9/11)
(pMTR11.32)	2nd	18	0	18 (7/11)
RF322 repeats with functional CENP-B boxes (69 kb)	1st	14	0	14 (5/9)
(pRF322B.192) Synthetic alpha repeats	2nd	21	0	21 (3/18)
RF322 repeats without CENP-B boxes (69 kb)	1st	11	0	11 (3/8)
(pRF322L.192)	2nd	11	0	11 (2/9)

[a]Chromosomal events were determined according to the predominant pattern (>50%) of introduced DNA containing synthetic α21-I 11mer alphoid repeat units or synthetic RF322 repeats, using FISH analysis of chromosome spreads. These patterns were counted in cases in which all three signals, DNA (DAPI), BAC, and α21-I alphoid signals, were colocalized. A χ^2 test of the predominant chromosomal events between pWTR11.32 and other cell lines with introduced plasmids showed highly significant P values (<0.0002).

minimal sequence requirement for de novo MAC formation: the telomeres

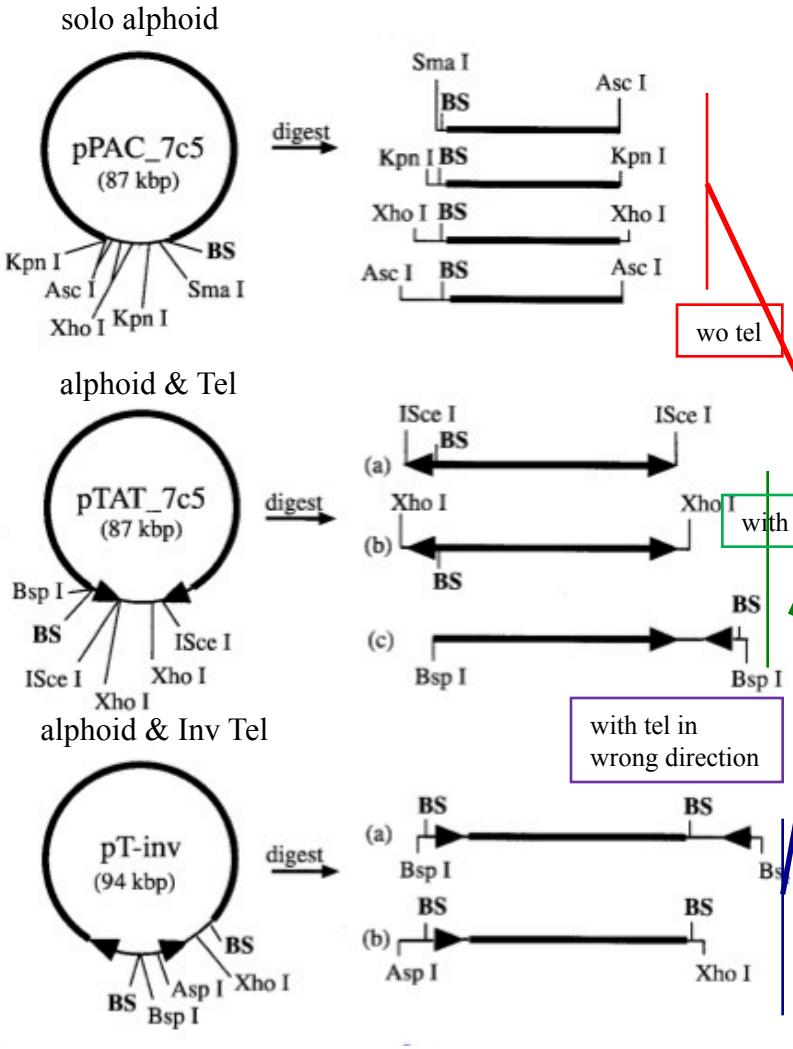


Table 1. MAC formation in HT1080 cells with the different PAC constructs shown in Figure 1

MAC in >50% delle cellule

MAC+ (%) for each of the positive clones

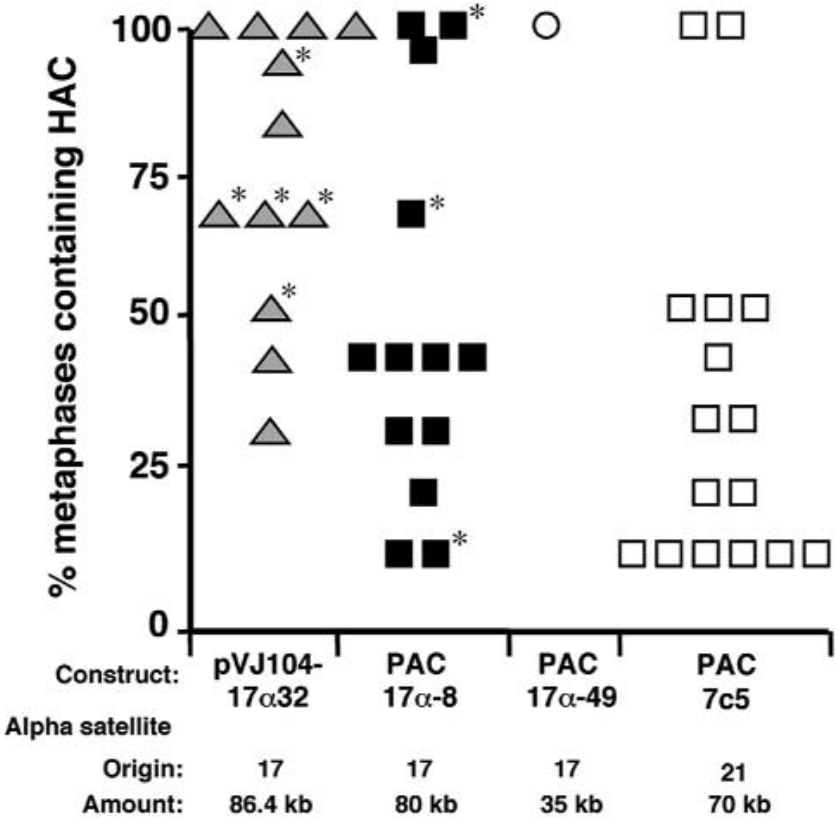
Construct	(L)	High frequency MAC formation	MAC frequencies (%)
I	(3)	10/21	100, 97, 96, 87, 80, 80, 80, 60, 50, 50
II	(4)	1/45	100
III	(3)	16/25	100, 100, 92, 87, 87, 87, 84, 77, 76, 72, 65, 63, 63, 60, 60, 50
IV (a)	(3)	6/26	90, 83, 80, 63, 57, 55
(b)	(2)	14/24	96, 93, 92, 85, 84, 83, 83, 80, 80, 77, 76, 73, 68, 64
(c)	(1)	6/10	96, 92, 85, 75, 73, 65
VI (a)	(1)	4/10	96, 84, 80, 68
(b)	(1)	2/11	100, 50
pac F15	(1)	0/10	

(L), number of lipofections carried out for each construct. Construct V was not used. High frequency MAC formation is the number of cell lines in which >50% of the cells contained a MAC (visible by FISH) out of the total number of cell lines analysed. MAC frequency (%) indicates the percentage of cells containing a MAC in each of the positive cell lines. A χ^2 test of the significance of the results gave a value of 22.8 ($P = 0.002$). Omitting four experiments where numbers were low reduced this χ^2 value to 14.4 ($P = 0.006$), still a highly significant value.

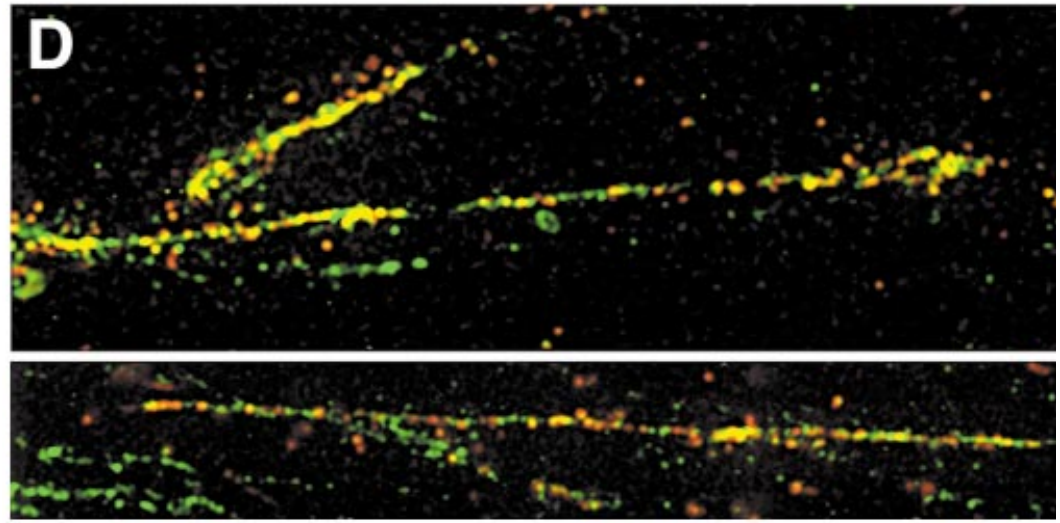
thick line, 70 kb of a21-1 alphoid repeats
thin line, vector
arrowheads, telomere arrays (800 bp)
BS, blasticidine S-methylase selectable marker

telomere sequences appear to augment the frequency of *de novo* chromosome formation but are dispensable

The length of the input array is also an important determinant, as reduction of the chromosome-17-based array from 80 kb to 35 kb reduced the frequency of HAC formation



Proportion of metaphase spreads containing a HAC within clonal lines generated from pVJ104-17□32, PAC17□-8, PAC17□-49, or PAC7c5. Asterisk (*) indicates lines with a high frequency of HAC formation also associated with an integration into a recipient chromosome



Stretched DNA fibers hybridizing with a BAC vector probe (red) and D17Z1 probe (green), indicating that the HACs are composed of amplified copies of the input DNA. Stretched fibers from the endogenous chromosome 17 □-satellite are those that hybridize exclusively with the D17Z1 probe.

de novo MAC
sequence requirement and structural features

- DNA alfoide del chr 7 e del 21
- almeno 80 kb di repeats clonati in un vettore circolare contenete un marcatore selezionabile
- I costrutti vengono introdotti nella linea HT1080 (cellule di fibroblasti); altre cellule permissive per la formazione dei minicromosomi non sono state descritte

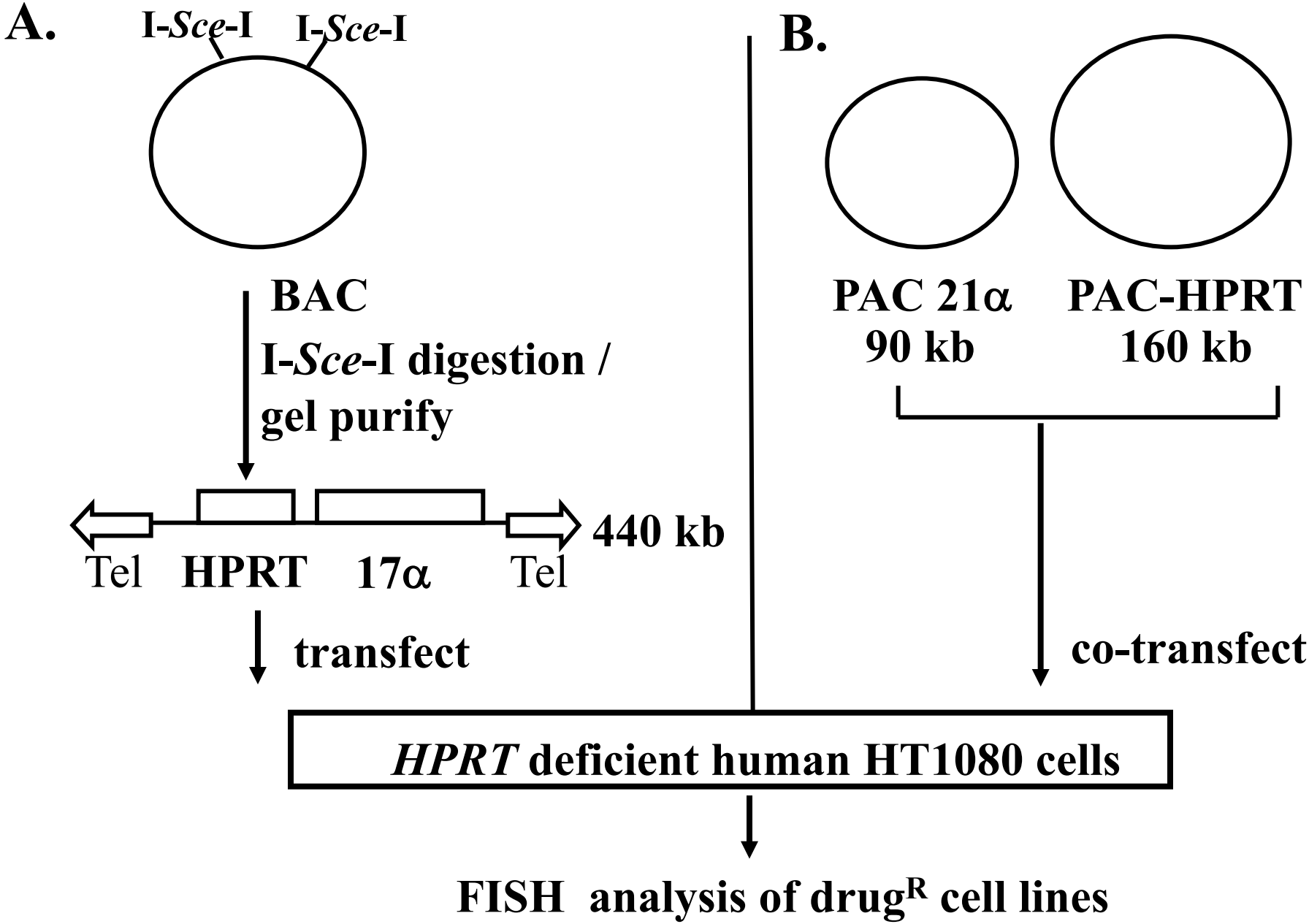
struttura

- piccole dimensioni
- contenetnti esclusivamente DNA esogeno (ovvero introdotto nella cellula tramite trasfezione)
- non contenevano DNA endogeno (ovvero appartenente alla cellula ospite)
- prevalentemente circolari
- la presenza di telomeri non influenza la formazione del cromosoma
- il Dna alfoide è alternato al DNA del vettore nel minicromosoma

how to insert a gene into de novo MAC

linked or un-linked constructs

De novo MAC terapeutici



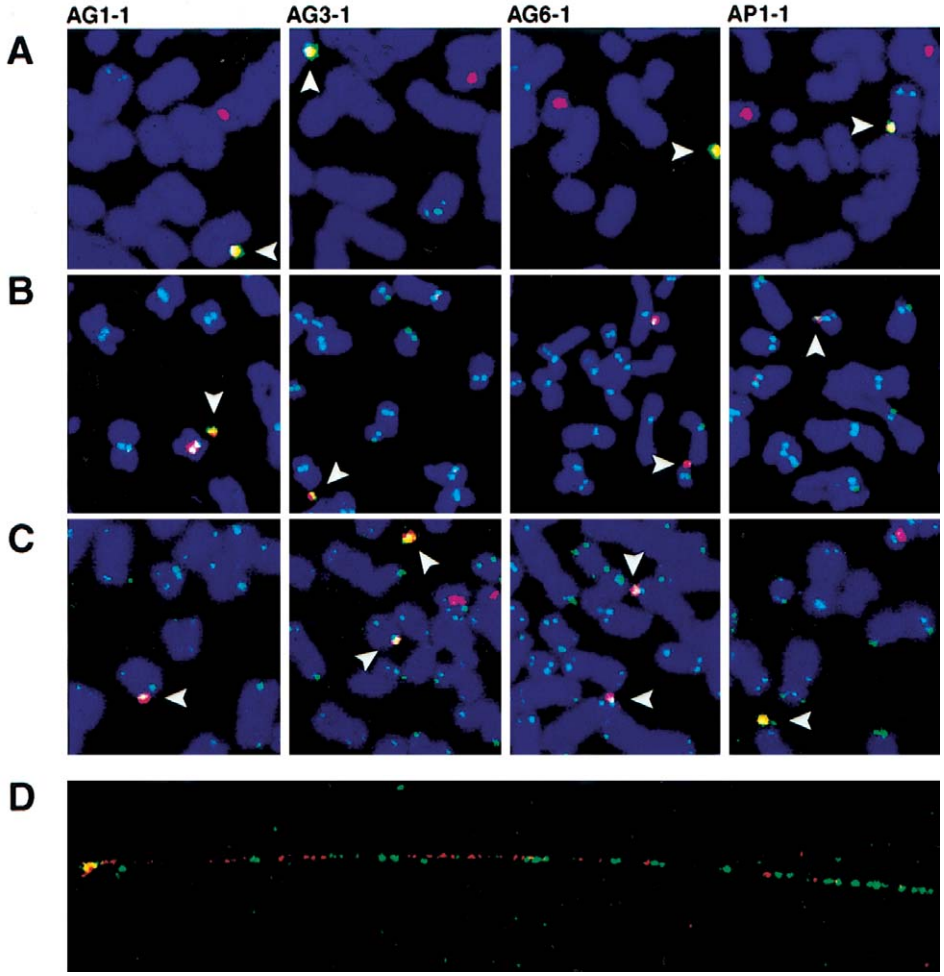
Mejia JE et al.,2001 *Am.J.Hum.Genet.*69:315-326

Grimes BR et al., 2001 Embo Rep.2:910-914

De novo HPRT-HAC obtained by lipofection of the linearized HPRT-BAC

De novo MAC terapeutici

four minichromosome cell lines—AG1-1, AG3-1, AG6-1, and AP1-1



(A), 17a (red) and DNA probe for the *HPRT1* locus (green)

(B)17a (red) and antibody to CENP-C (green), a marker of functional centromeres

(C); 17a (red) – and human-telomeric–DNA probe (green).

(D) Dual hybridization of 17 a (red *signal)* and *HPRT1-locus* (green *signal)* probes to stretched DNA fibers from the AG6-1 cell line. The signals show that both sequences alternate on the minichromosome, as consecutive segments of irregular size. Gaps along the minichromosome fiber are expected where sequences are not represented in the probes used

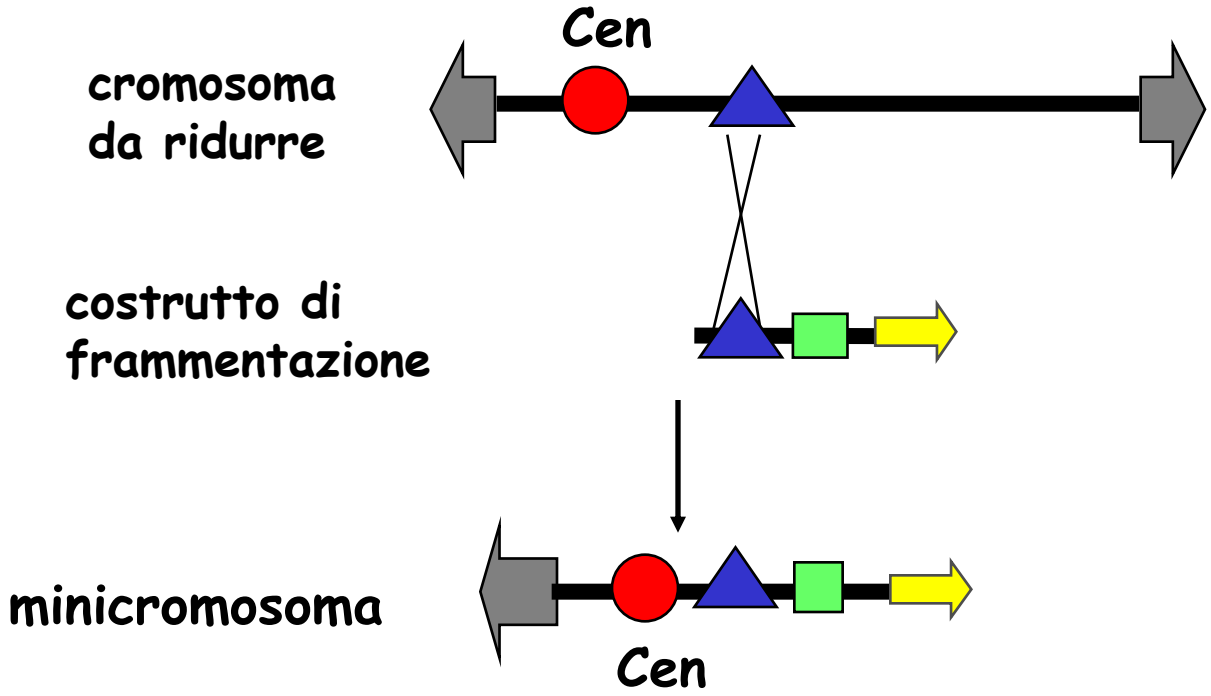
Meja 2001 Am. J. Hum. Genet. 69:315–326, 2001

the top down approach

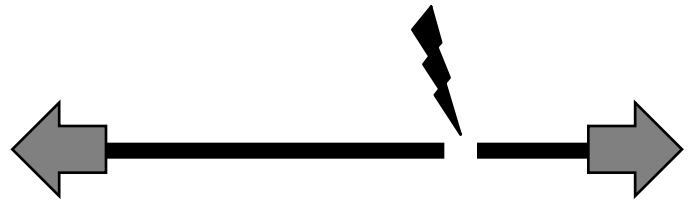
a pruning procedure that makes the chromosome reed of dispensable sequences/elements

TOP-DOWN

A) FRAMMENTAZIONE TELOMERICA: utilizzo di plasmidi lineari contenenti:
-sequenze telomeriche umane
-gene per la selezione
-sequenze "target" per la localizzazione del punto di rottura

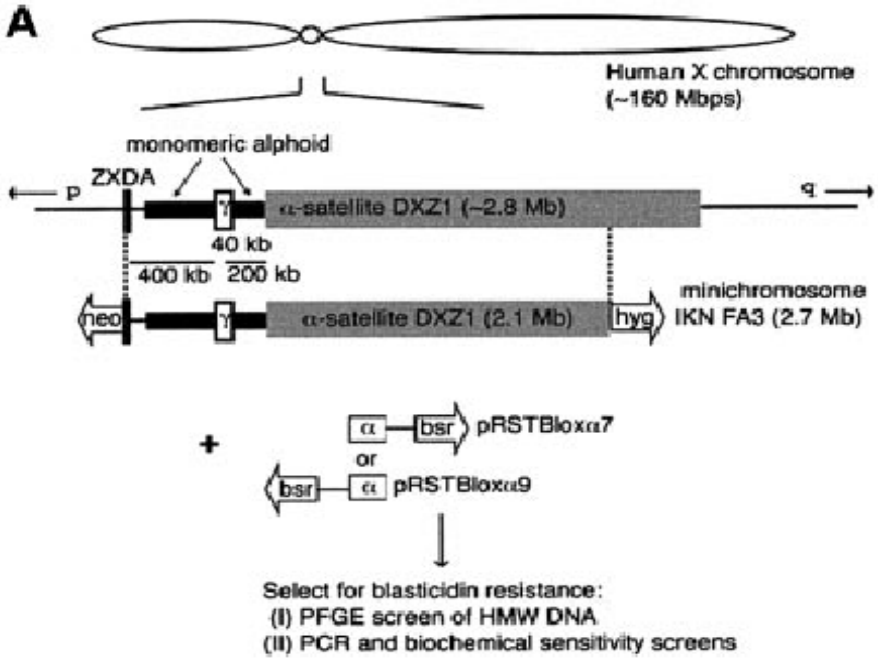


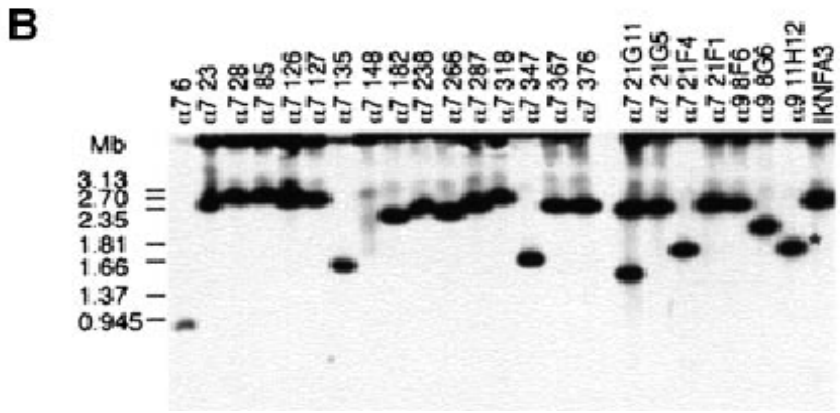
B) IRRAGGIAMENTO DI CROMOSOMI NATURALI

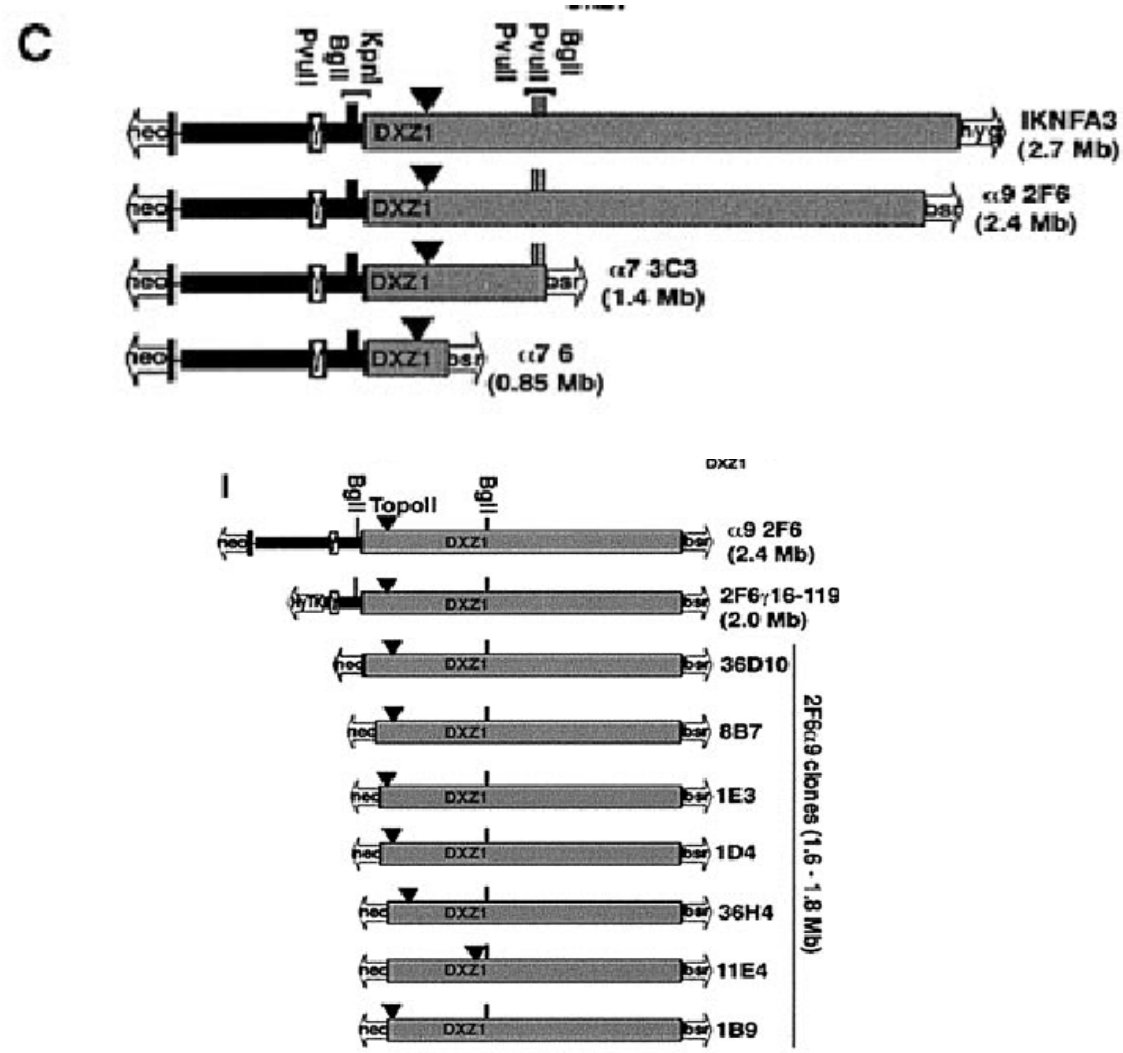


Frammentazione del cromosoma X umano

(frammentazione telomerica)







Spence et al., 2002 EMBO J 19, 5269-5280

Proprietà strutturali

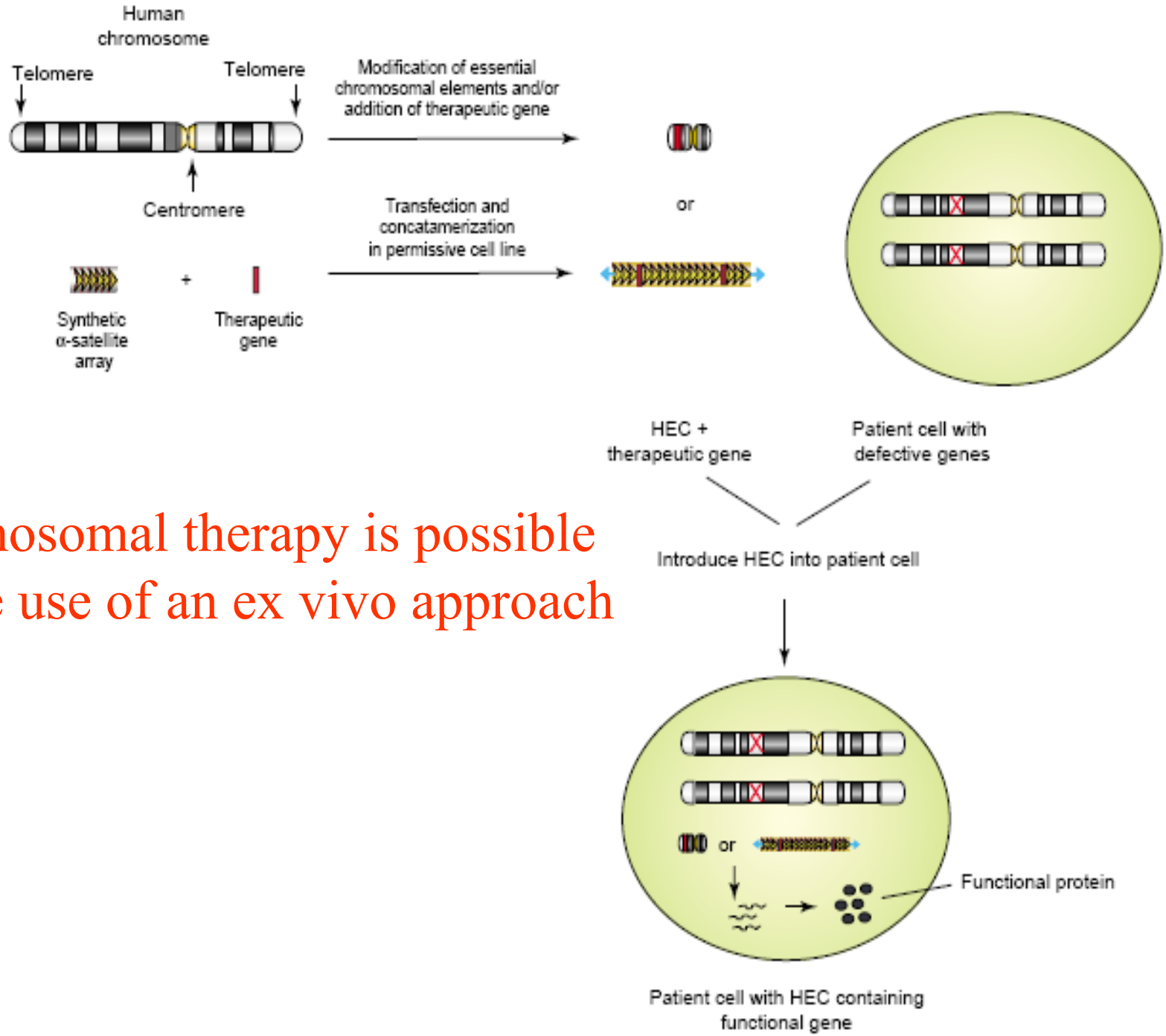
minicromosomi *de novo*

• molecole circolari/*circular*
• dimensioni comprese tra le 5-10 Mb/*size 5-10 Mb*
• struttura non determinabile a priori a causa di eventi di multimerizzazione a carico del DNA trasferito/*the structure is not simply related to the input construct*
• la formazione di cromosomi *de novo* potrebbe essere associata a riarrangiamenti genomici della cellula ospite/*de novo chromosome formation may be associated with genome rearrangements*

minicromosomi "top down"

• struttura lineare con telomeri funzionali/*linear*
• dimensioni che vanno dalle poche centinaia di kb fino a 5-10 Mb/*size from few hundreds kb to 5-10 Mb*
• struttura conforme a quella del cromosoma parentale/*the structure is strictly related to the parental chromosome*

how to insert a MAC into target cells



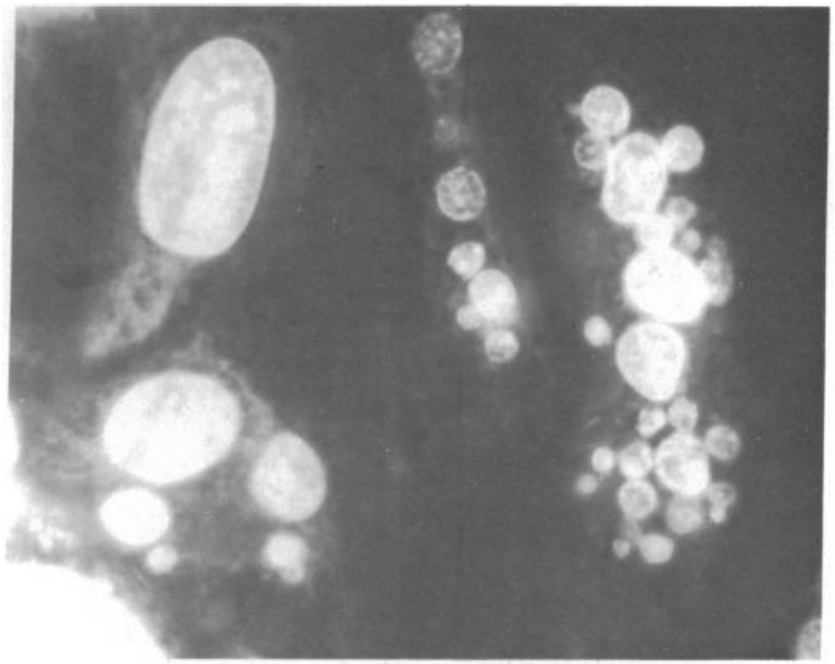
chromosomal therapy is possible by the use of an ex vivo approach

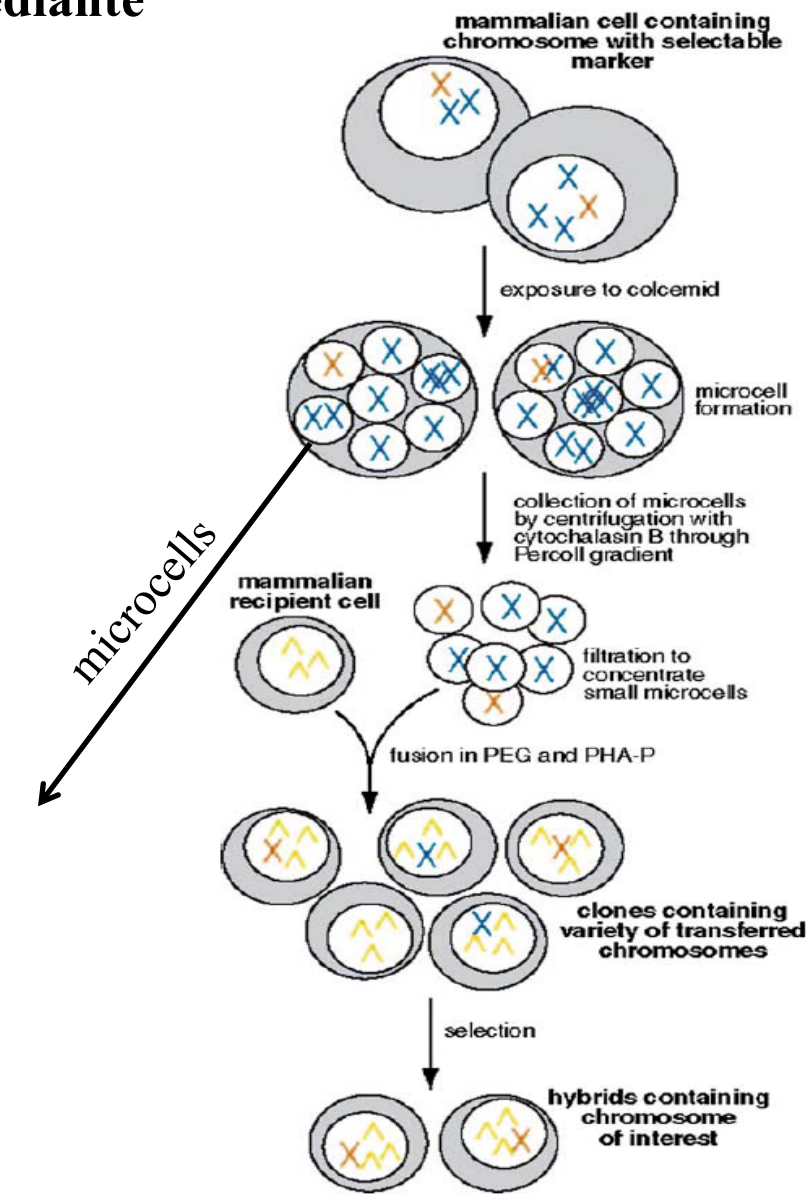
DT40 lynphocyte cell line

- this cell line is derived from chicken lynphocytes
- it is used to assemble hybrids with chromosomes from other species, bumans included
- it is useful for chromosome engineering since these cells exhibit efficient homologous recombination that allows targeting of the sequence of interest into specific regions
- DT40 cells, after prolonged cell cycle block (typically colcemide for 16-18 hr) form microcells

Sistemi di trasferimento dei MAC in cellule umane per terapia genica

- **Trasfezione di minicromosomi purificati mediante FACS sorter**
- **Microiniezione**
- **Trasferimento cromosomico mediato dalle microcellule (MMCT)**





Frammentazione del cromosoma 21 umano (frammentazione telomerica)

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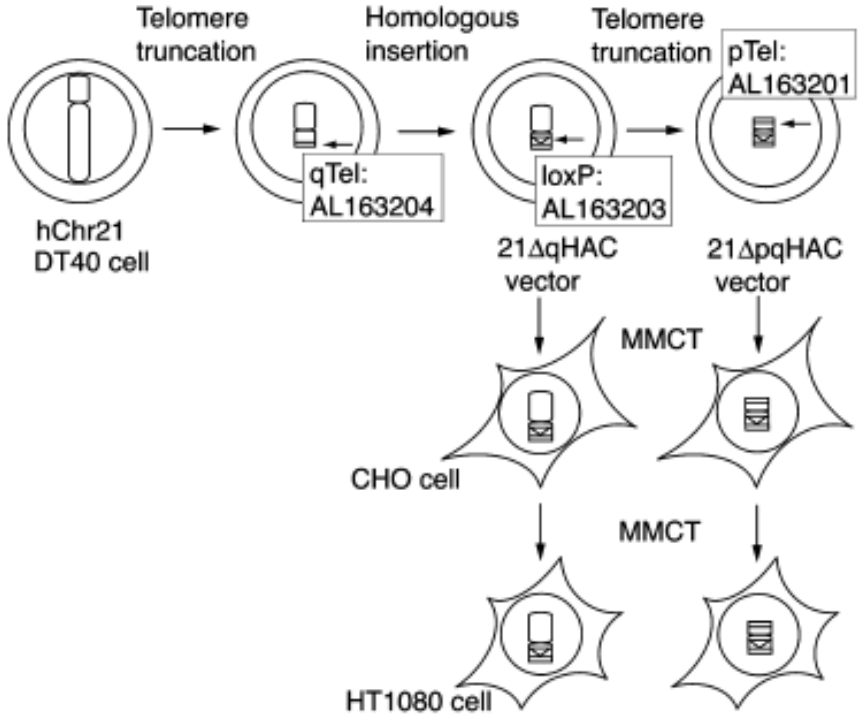
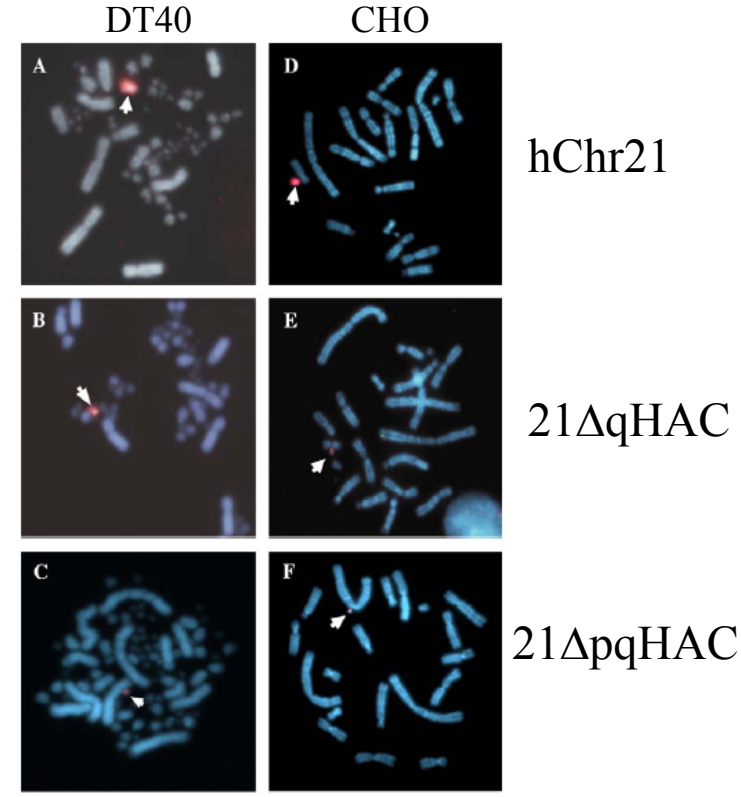
Biochemical and Biophysical Research Communications 321 (2004) 280–290
BBRC
www.elsevier.com/locate/ybbrc

Construction of a novel human artificial chromosome vector for gene delivery

Motonobu Katoh [a], Fumiaki Ayabe [b], Satoko Norikane [c], Teruaki Okada [d], Hiroshi Masumoto [d], Shin-ichi Horike [c,1], Yasuaki Shirayoshi [c], Mitsuo Oshimura [a,b,c,*]

[a] *Department of Human Genome Sciences (Kirin Brewery), Graduate School of Medical Science, Tottori University, 86 Nishimachi, Yonago, Tottori 683-8503, Japan*
[b] *Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, Tottori University, 86 Nishimachi, Yonago, Tottori 683-8503, Japan*
[c] *Department of Molecular and Cell Genetics, Graduate School of Medical Science, Tottori University, 86 Nishimachi, Yonago, Tottori 683-8503, Japan*
[d] *Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan*





Gene Therapy (2005) 12, 852–856

www.nature.com/gt

BRIEF COMMUNICATION

Human artificial chromosome (HAC) vector provides long-term therapeutic transgene expression in normal human primary fibroblasts

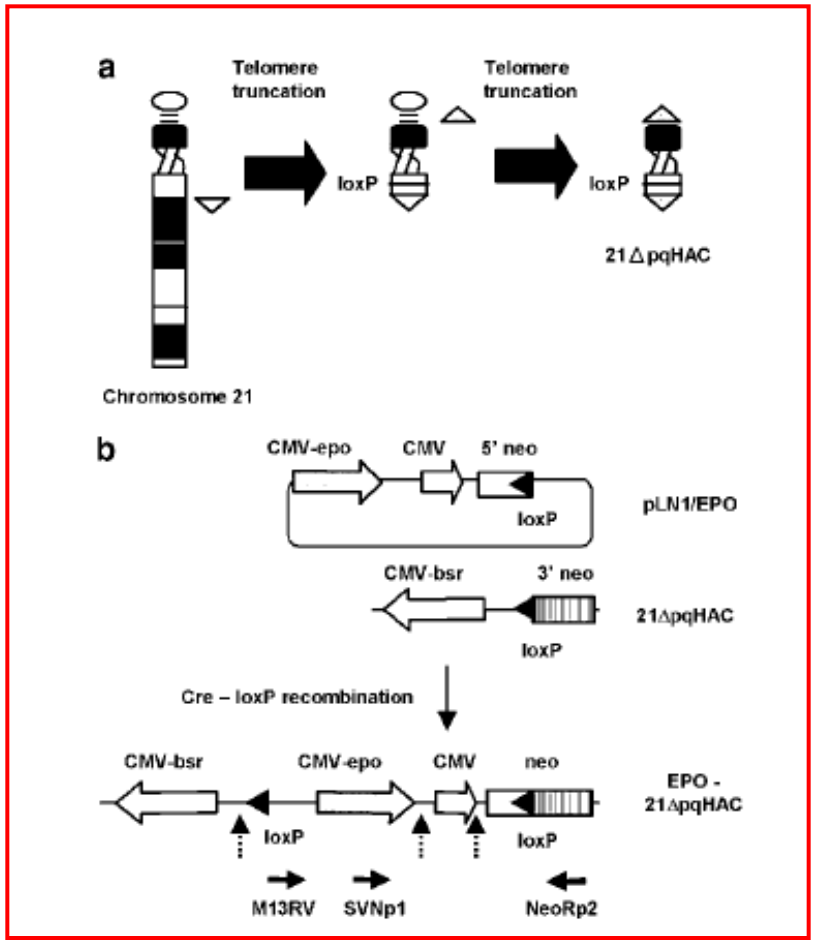
M Kakeda[1], M Hiratsuka[1,2], K Nagata[1], Y Kuroiwa[1], M Kakitani[1], M Katoh[2], M Oshimura[2] and K Tomizuka[1,2]

[1]*Pharmaceutical Research La*

[2]*Biomedical Science, Institut*

Nishimachi, Yonago-shi, Tott

21ΔpqHAC + epo cDNA constrct



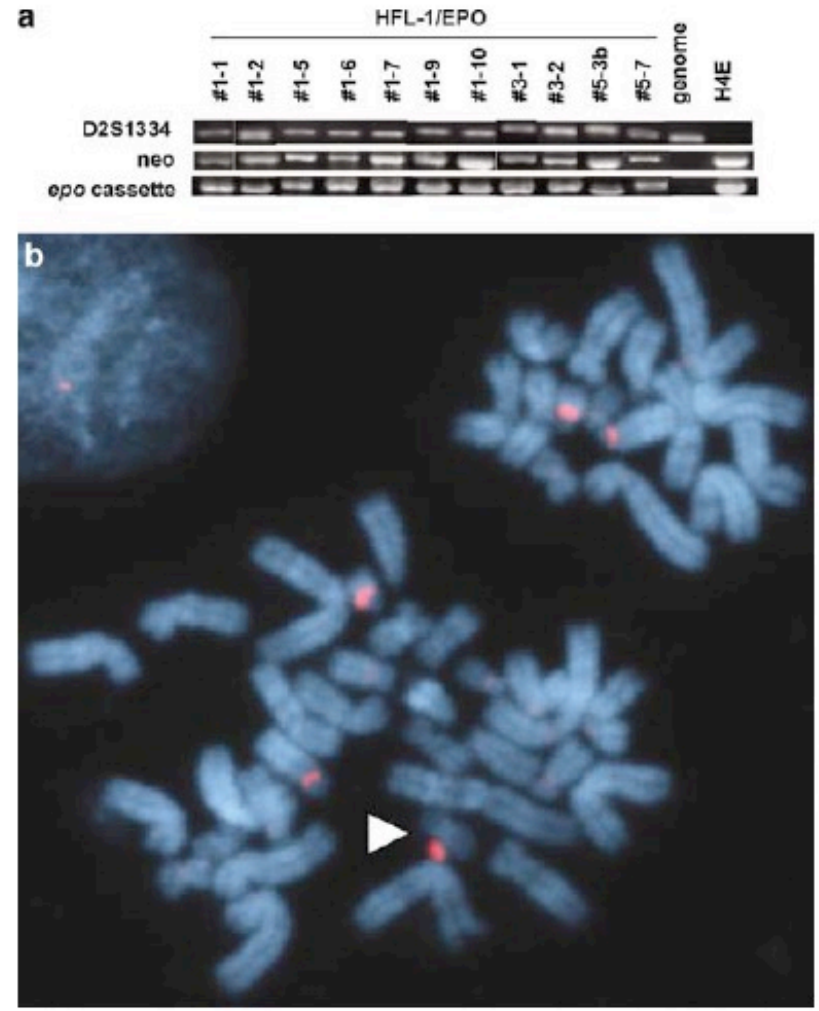
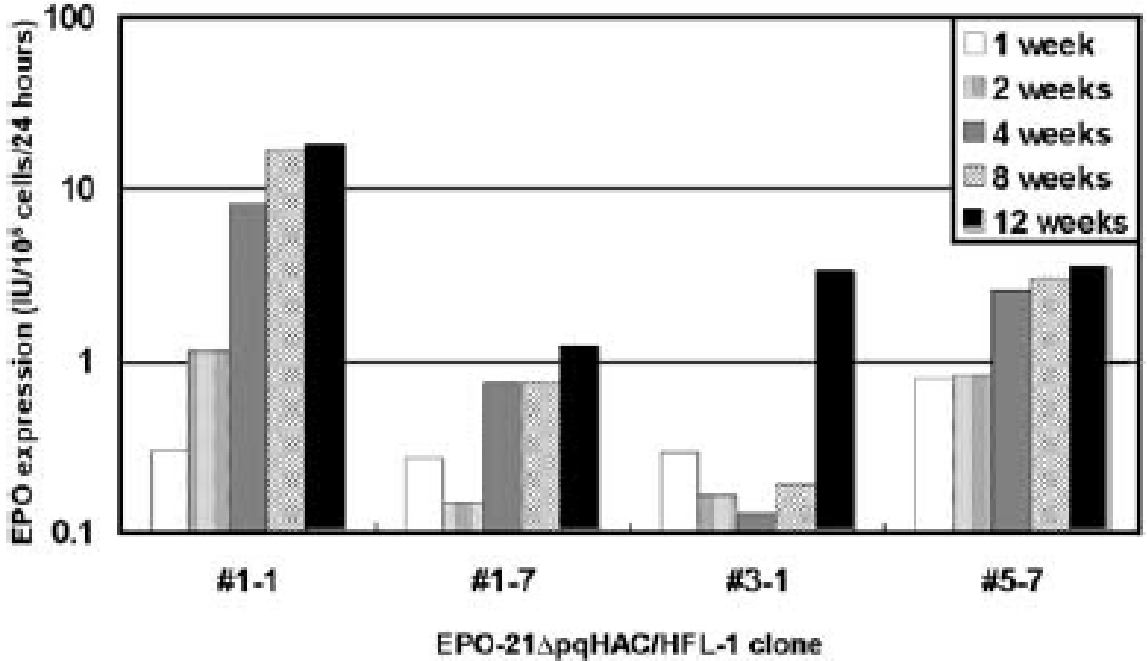


Table 1 Mitotic stability of the EPO-21ΔpqHAC in normal hPFs

Clone	*Passage*	*Number of metaphase*	*Number of 21αDNA signals/cell*			*Retention rate of HAC (%)*	*Loss rate (R)*
			4+0	*4+1*	*4+2*		
#1-1	Initial	38	3	35	0	92.1	
#1-7	Initial	50	0	28	22	100.0	
#3-1	Initial	57	3	54	0	94.7	
#5-7	Initial	34	4	30	0	88.2	
#1-1	9	29	10	19	0	65.5	0.023
#1-7	6	44	4	22	18	90.9	0.0094
#3-1	6	40	9	31	0	77.5	0.019
#5-7	6	37	11	26	0	70.2	0.022

21αDNA[14]; a human alphoid DNA probe from the centromeric region of chromosome 21. Mitotic-loss rates of the EPO-21ΔpqHAC vector were calculated by the following formula: $N_n = N_0 \times (1-R)^n$, where N is the retention rate after 'n' generations, N_0 is the retention rate at day 0 and R is the loss rate per generation. Passages 6 and 9 were equivalent to 10 and 14 generations, respectively.



long term Epo expression

Kakeda et al., 2005

A Novel Human Artificial Chromosome Vector Provides Effective Cell Lineage–Specific Transgene Expression in Human Mesenchymal Stem Cells

Xianying Ren, Motonobu Katoh, Hidetoshi Hoshiya, Akihiro Kurimasa, Toshiaki Inoue, Fumiaki Ayabe, Kotaro Shibata, Junya Toguchida and Mitsuo Oshimura

Stem Cells 2005;23;1608-1616; originally published online Sep 1, 2005;
DOI: 10.1634/stemcells.2005-0021

Mesenchymal stem cells (MSCs) hold promise for use in adult stem cell–mediated gene therapy. One of the major aims of stem cell–mediated gene therapy is to develop vectors that will allow appropriate levels of expression of therapeutic genes along differentiation under physiological regulation of the specialized cells. Human artificial chromosomes (HACs) are stably maintained as independent chromosomes in host cells and should be free from potential insertional mutagenesis problems of conventional transgenes. Therefore, HACs have been proposed as alternative implements to cell-mediated gene therapy. Previously, we constructed a novel HAC, **termed 21 Δpq HAC, with a loxP site in which circular DNA can be reproducibly inserted by the Cre/loxP system.**

We here assessed the feasibility of lineage-specific transgene expression by the 21Δpq HAC vector using an in vitro differentiation system with an MSC cell line, hiMSCs, which has potential for osteogenic, chondrogenic, and adipogenic differentiation.

An enhanced green fluorescent protein (EGFP) gene driven by a promoter for osteogenic lineage-specific *osteopontin* (OPN) gene was inserted onto the 21 Δpq HAC and then transferred into hiMSC.

The expression cassette was flanked by the chicken HS4 insulators to block promoter interference from adjacent drug-resistant genes.

The *EGFP* gene was specifically expressed in the hiMSC that differentiated into osteocytes in coordination with the transcription of endogenous *OPN* gene but was not expressed after adipogenic differentiation induction or in noninduction culture.

These results suggest that use of the HAC vector is suitable for regulated expression of transgenes in stem cell-mediated gene therapy

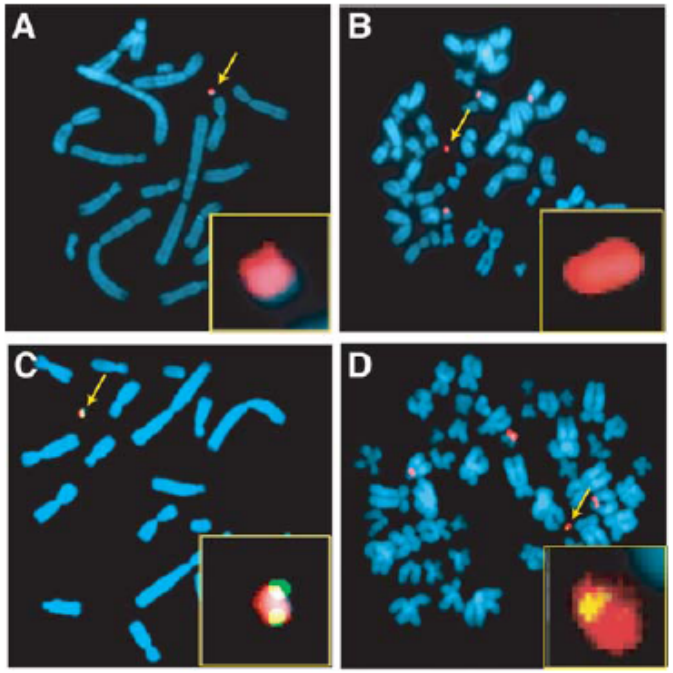
We here assessed the feasibility of lineage-specific transgene expression by the 21Δpq HAC vector using an in vitro differentiation system with an MSC cell line, hiMSCs, which has potential for osteogenic, chondrogenic, and adipogenic differentiation.

FISH analysis of CHO and hiMSC hybrid cells carrying the 21Δpq HAC vectors or 21Δpq HAC/In-OPN-EGFP vector.

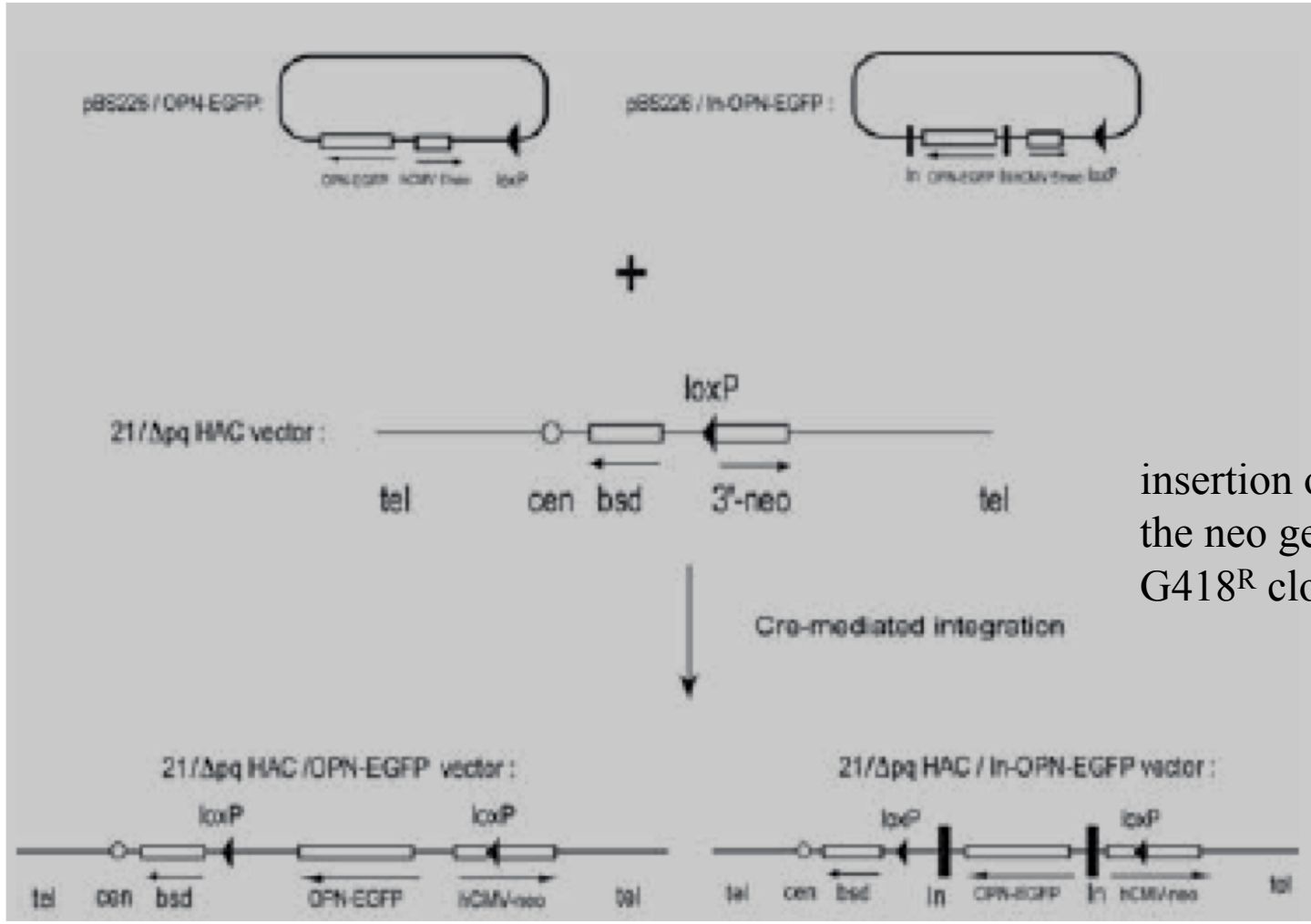
CHO 21Dpq (A,C) hiMSC 21Dpq (B,D)

alphoid probe

GFP probe



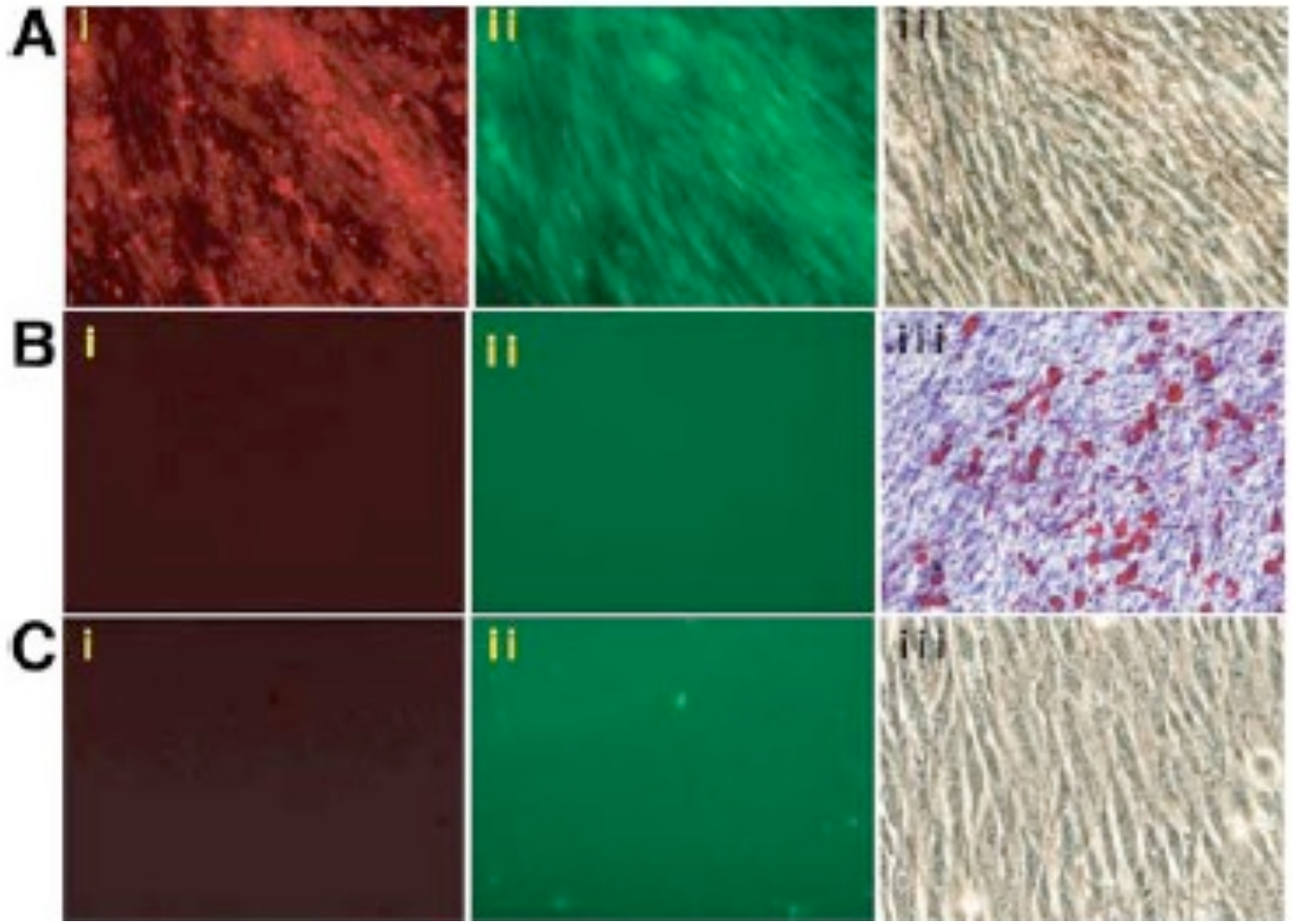
21 Δpq HAC, with a loxP site in which circular DNA can be reproducibly inserted by the Cre/loxP system. An enhanced green fluorescent protein (EGFP) gene driven by a promoter for osteogenic lineage-specific *osteopontin* (OPN) gene was inserted onto the 21 Δpq HAC and then transferred into hiMSC.



insertion of the vector reconstitutes the neo gene and allows selection of $G418^R$ clones

Lineage-specific EGFP expression in hiMSC hybrids con- taining 21Δpq HAC/In-OPN-EGFP vector after osteogenic differentiation

A (i): Detection of red fluorescence produced by alkaline phosphatase activity (osteogenic differentiation); (ii): detection of green fluorescence of EGFP(HAC); (iii): phase-contrast microscopic. **B**. adiponenic differentiation, B(iii)lipid vacuoles in red. **(C)** control culture without differentiation induction.



Representative fluorescence and phase-contrast microscopic view of hiMSC hybrids.

Cross panel (A) after osteogenic differentiation, GFP on

(B) after adipogenic differentiation, GFP off

(C) control culture without differentiation induction
GFP off

Ren et al.,2005

MAC *in vivo*?

from the cell to the organism

Cloned transchromosomic calves producing human immunoglobulin

Yoshimi Kuroiwa[1], Poothappillai Kasinathan[2], Yoon J. Choi[3], Rizwan Naeem[4], Kazuma Tomizuka[1], Eddie J. Sullivan[2], Jason G. Knott[2], Anae Duteau[3], Richard A. Goldsby[3], Barbara A. Osborne[5], Isao Ishida[1]*, and James M. Robl[2]*

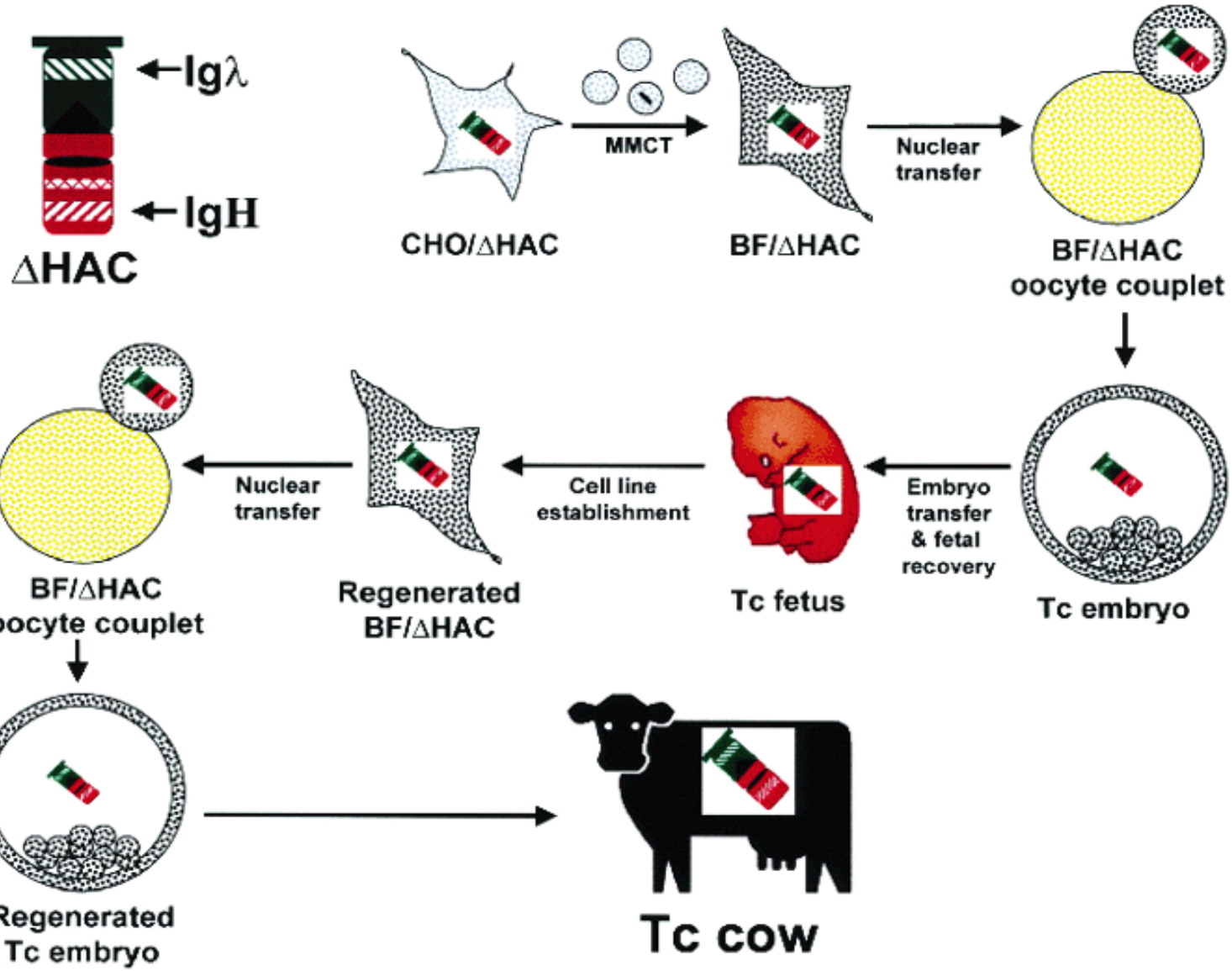
Human polyclonal antibodies (hPABs) are useful therapeutics, but because they are available only from human donors, their supply and application is limited.
To address this need, we prepared a human artificial chromosome (HAC) vector containing the entire unrearranged sequences of the human immunoglobulin (h*Ig*) heavy- chain (*H*) and lambda (λ) light-chain loci.
1.The HAC vector was introduced into bovine primary fetal fibroblasts using a microcell-mediated chromosome transfer (MMCT) approach.
2.Primary selection was carried out, and the cells were used to produce cloned bovine fetuses.
3.Secondary selection was done on the regenerated fetal cell lines, which were then used to produce four healthy transchromosomic (Tc) calves.

The HAC was retained at a high rate (78–100% of cells) in calves and the h*Ig* loci underwent rearrangement and expressed diversified transcripts. **Human immunoglobulin proteins were detected in the blood of newborn calves.** The production of Tc calves is an important step in the development of a system for producing therapeutic hPABs.

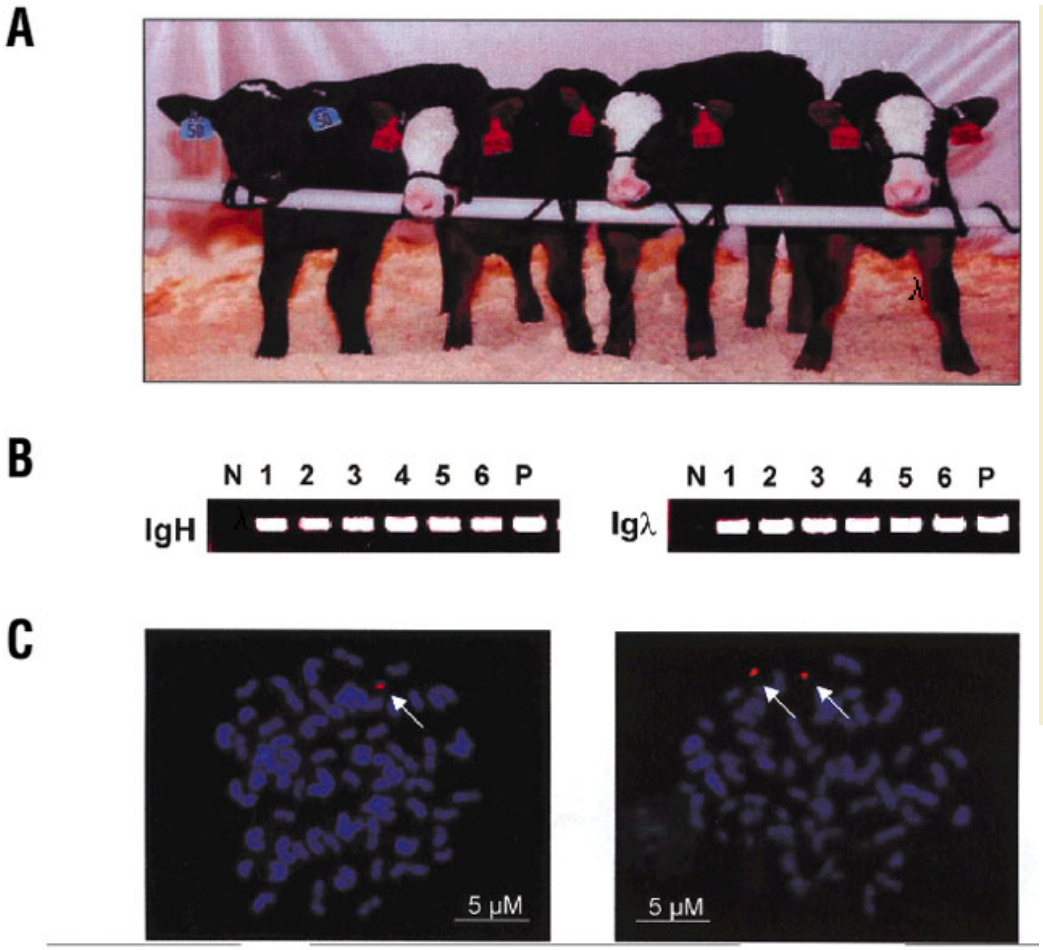
Applicazioni dei MAC in vivo

"Cloned transchromosomic calves producing human immunoglobulin"

human Ig loci13 (1–1.5 Mb for each locus)



Kuroiwa et al, Nature Biotechnology 20, 889-894 (2002)



.
(A) Four cloned Tc calves: male calf (50) from cell line 6045 and female calves (1064, 1065, 1066) from cell line 5968. **(B)** Genomic PCR of *IgH* and *Ig* loci in PBLs from cloned Tc calves and controls: calf 1064 (lane 1), 1065 (lane 2), 1066 (lane 3), 50 (lane 4), 1067 (lane 5), and 1068 (lane 6). Both human *IgH* and *Ig* loci were detected by genomic PCR in all the Tc calves and positive-control human liver DNA (lane P), but not in a negative-control nontransgenic calf (lane N). **(C)** FISH analysis in metaphase chromosome spreads in a cell showing a single signal and a cell showing a double signal. Arrows indicate location of HACs (red) among surrounding bovine chromosomes (blue). A single HAC per cell is introduced and retained in most cells (left panel); however, improper separation of chromatids at cell division may result in some cells having two microchromosomes (right panel) and some not having a microchromosome

- **l' analisi FISH ha evidenziato la presenza del vettore HAC, come cromosoma indipendente, nel 78-100% delle cellule**
- **non sono state rilevate differenze tra i linfociti del sangue periferico (91%) ed i fibroblasti (87%)**
- **l' analisi di RT-PCR ha evidenziato l' espressione dei geni umani *IgH* e *Igλ* nei linfociti del sangue periferico**

therapeutic HAC

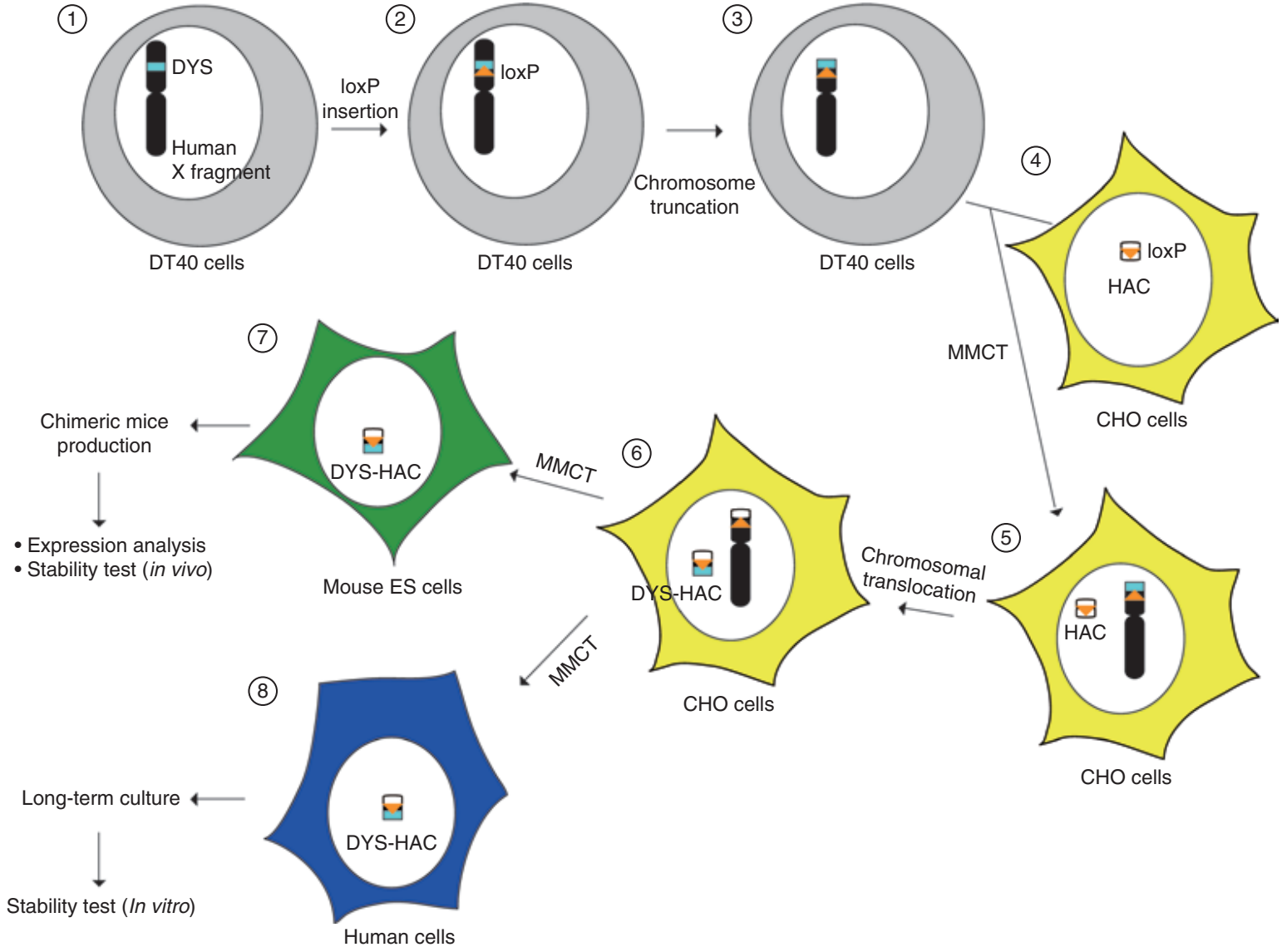
A highly Stable and Nonintegrated Human Artificial Chromosome (HAC) Containing the 2.4 Mb Entire Human Dystrophin Gene

Hidetoshi Hoshiya[1], Yasuhiro Kazuki[1], Satoshi Abe[1], Masato Takiguchi[1], Naoyo Kajitani[1], Yoshinori Watanabe[1], Toko Yoshino[2], Yasuaki Shirayoshi[3], Katsumi Higaki[2], Graziella Messina[4], Giulio Cossu[4] and Mitsuo Oshimura[1]

Molecular Therapy 2009

Episomal vector with the capacity to deliver a large gene containing all the critical regulatory elements is ideal for gene therapy. Human artificial chromosomes (HACs) have the capacity to deliver an extremely large genetic region to host cells without integration into the host genome, thus preventing possible insertional mutagen- esis and genomic instability. Duchenne muscular dystro- phy (DMD) is caused by mutation in the extremely large dystrophin gene (2.4 Mb). We herein report the devel- opment of a HAC vector containing the entire human dystrophin gene (DYS-HAC) that is stably maintained in mice and human immortalized mesenchymal stem cells (hiMSCs). The DYS-HAC was transferred to mouse embryonic stem (ES) cells, and isoforms of the DYS- HAC-derived human dystrophin in the chimeric mice generated from the ES cells were correctly expressed in tissue-specific manner. Thus, this HAC vector contain- ing the entire dystrophin gene with its native regulatory elements is expected to be extremely useful for future gene and cell therapies of DMD.

schematic diagram of the construction of various cells containing the dYs-HAc vector



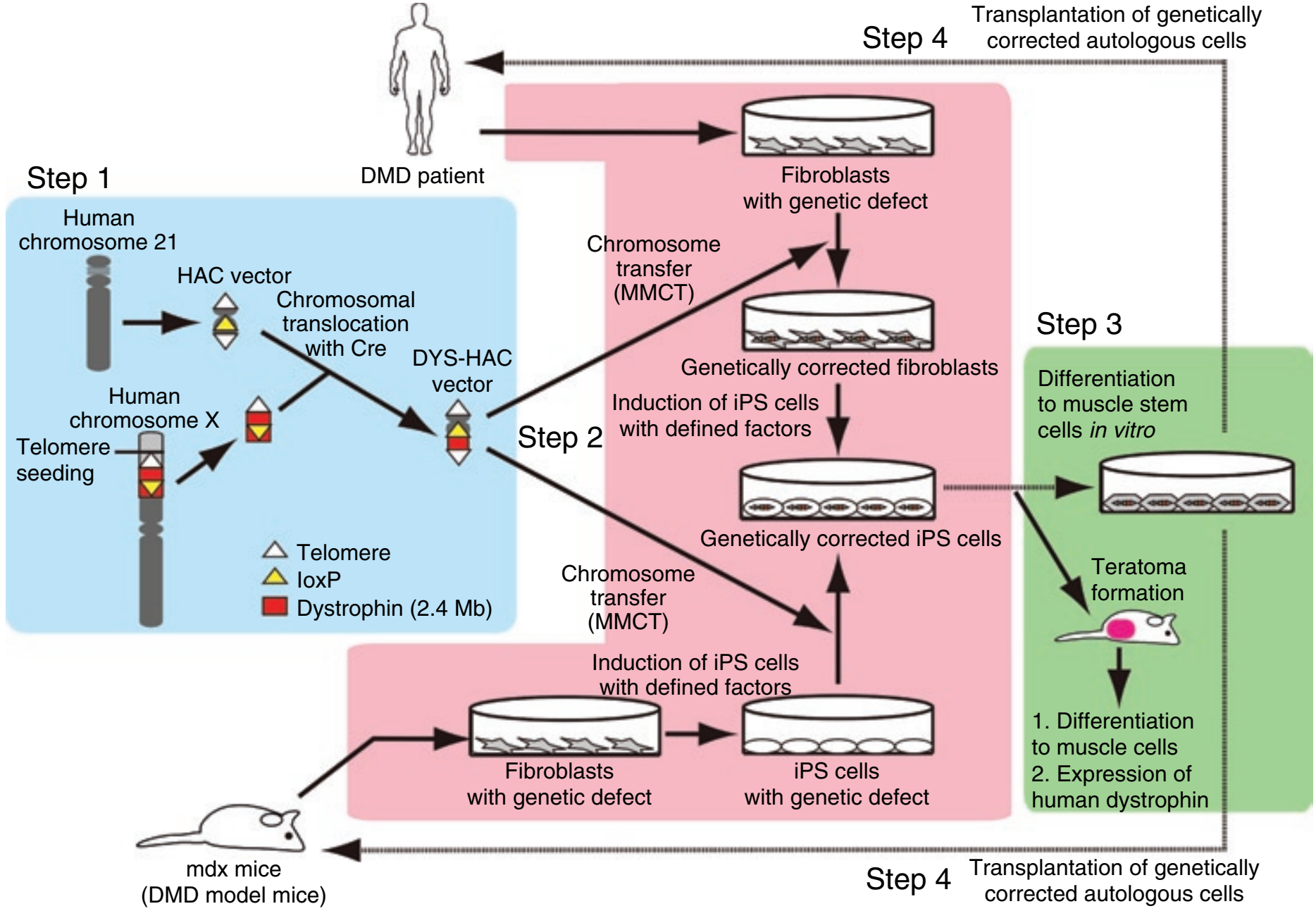
Molecular Therapy vol. 18 no. 2 feb. 2010

Complete Genetic Correction of iPS Cells From Duchenne Muscular Dystrophy

Yasuhiro Kazuki[1], Masaharu Hiratsuka[2], Masato Takiguchi[1], Mitsuhiko Osaki[1], Naoyo Kajitani[1], Hidetoshi Hoshiya[1], Kei Hiramatsu[1], Toko Yoshino[3], Kanako Kazuki[1], Chie Ishihara[1], Shoko Takehara[1], Katsumi Higaki[3], Masato Nakagawa[4,5], Kazutoshi Takahashi[4], Shinya Yamanaka[4–6] and Mitsuo Oshimura[1,7]

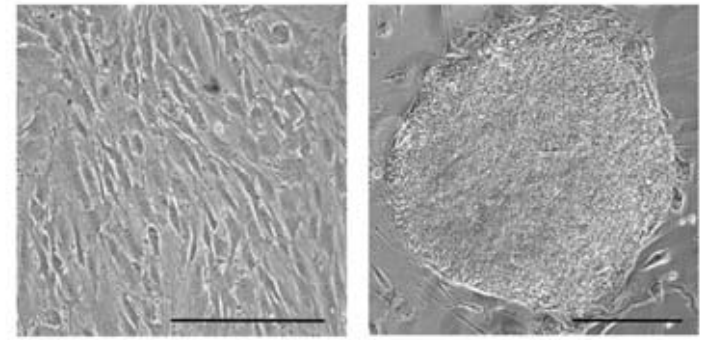
Human artificial chromosome (HAC) has several advantages as a gene therapy vector, including stable episomal maintenance that avoids insertional mutations and the ability to carry large gene inserts including the regula- tory elements. Induced pluripotent stem (iPS) cells have great potential for gene therapy, as such cells can be generated from the individual's own tissues, and when reintroduced can contribute to the specialized function of any tissue.

As a proof of concept, we show herein the complete correction of a genetic deficiency in iPS cells derived from Duchenne muscular dystrophy (DMD) model (mdx) mice and a human DMD patient using a HAC with a complete genomic dystrophin sequence (DYS-HAC). Deletion or mutation of dystrophin in iPS cells was corrected by transferring the DYS-HAC via microcell-mediated chromosome transfer (MMCT). DMD patient- and mdx-specific iPS cells with the DYS- HAC gave rise to differentiation of three germ layers in the teratoma, and human dystrophin expression was detected in muscle-like tissues. Furthermore, chimeric mice from mdx-iPS (DYS-HAC) cells were produced and DYS-HAC was detected in all tissues examined, with tissue-specific expression of dystrophin. Therefore, the combination of patient-specific iPS cells and HAC- containing defective genes represents a powerful tool for gene and cell therapies.

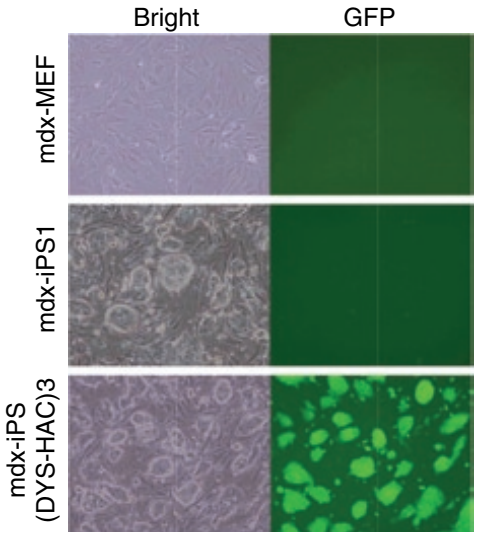

Step 4
Transplantation of genetically corrected autologous cells
DMD patient
Step 1
Human chromosome 21
HAC vector
Chromosomal translocation with Cre
DYS-HAC vector
Human chromosome X
Telomere seeding
Telomere
loxP
Dystrophin (2.4 Mb)
Fibroblasts with genetic defect
Chromosome transfer (MMCT)
Genetically corrected fibroblasts
Induction of iPS cells with defined factors
Step 2
Genetically corrected iPS cells
Step 3
Differentiation to muscle stem cells *in vitro*
Teratoma formation
1. Differentiation to muscle cells
2. Expression of human dystrophin
Chromosome transfer (MMCT)
Induction of iPS cells with defined factors
Fibroblasts with genetic defect
iPS cells with genetic defect
mdx mice (DMD model mice)
Step 4
Transplantation of genetically corrected autologous cells

iPS from mdx mice and correction of mdx defciency with DYS-HAC

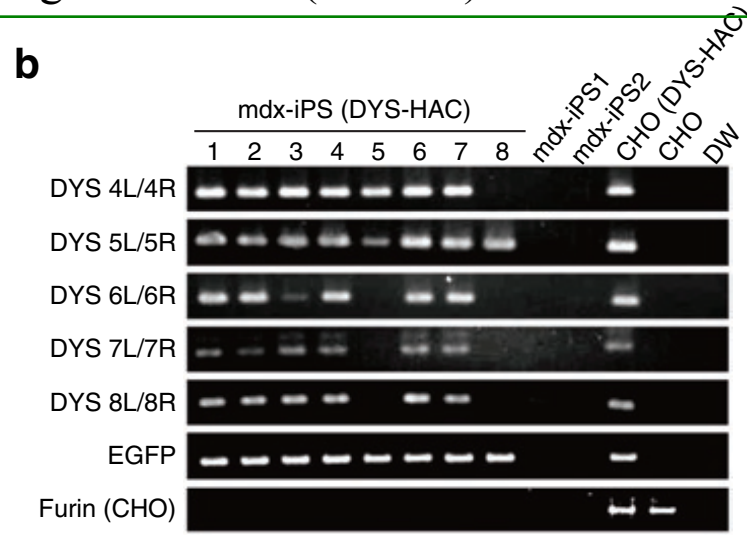
The mdx-iPS cells were induced from mdx mouse embryonic fibroblasts by retroviral infection of the three factors including Klf4, Sox2, and Oct4. Transduced fibroblasts from the mdx mice gave rise to ES cell– like colonies, and these colonies were isolated based on morphological criteria



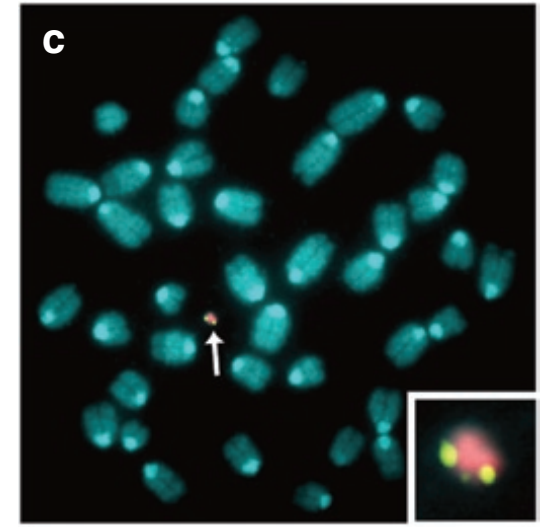
Microcells mediated gene transfer (MMCT) of DYS-HAC in mdx-iPS



(a) Morphology of mdx-MEF, mdx-iPS, and mdx-iPS (DYS-HAC) cells. Phase-contrast (left panel) and GFP-fluorescence (right panel) micrographs are shown

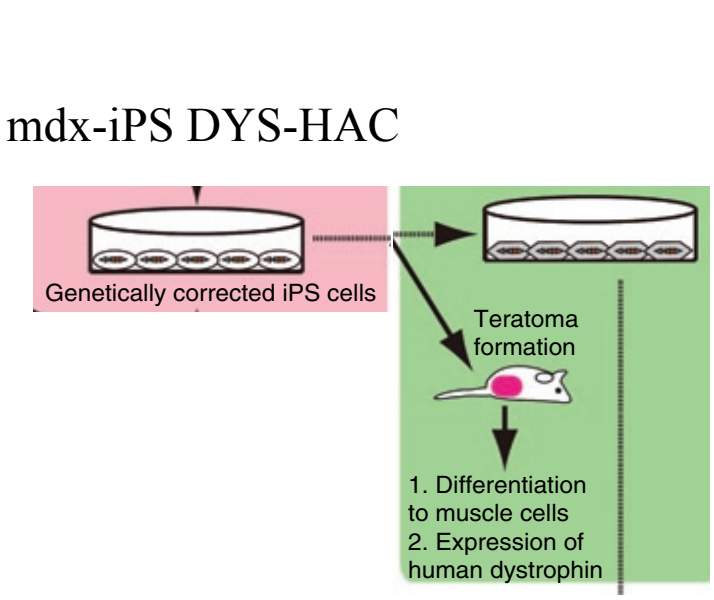


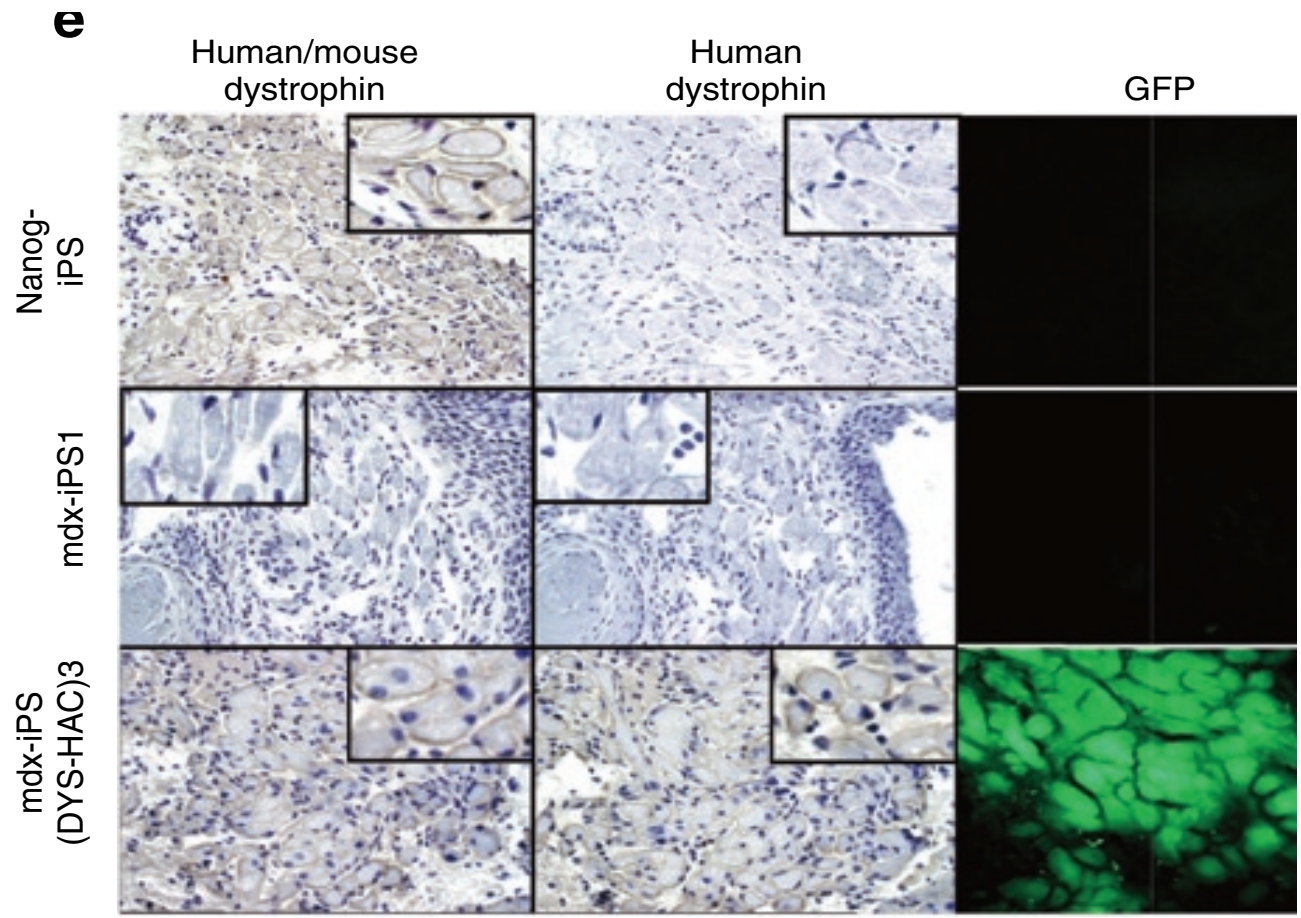
(b) Genomic PCR analyses for detecting DYS-HAC in mdx-iPS cells.



(c) FISH analyses for mdx-iPS (DYS-HAC) cells. An arrow indicates the DYS-HAC and the inset shows an enlarged image of the DYS-HAC.

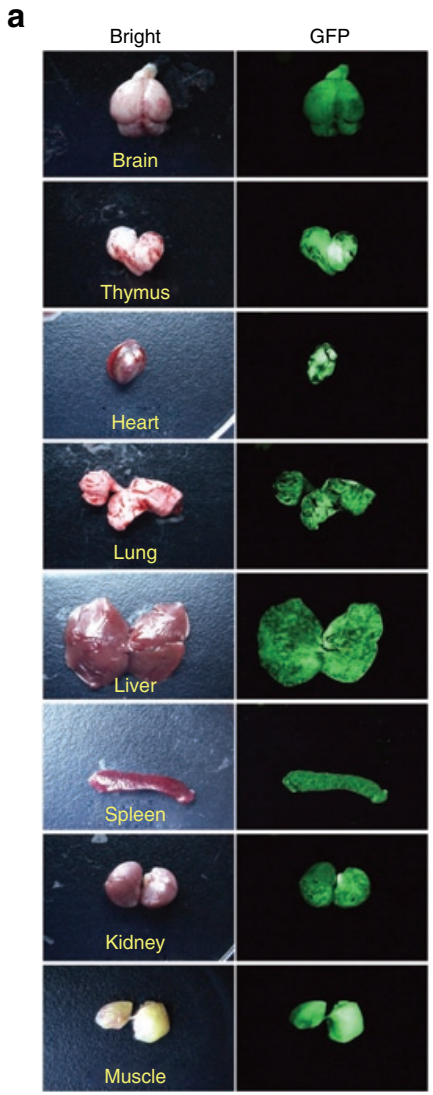
Expression of human dystrophin in muscle-like tissues of teratomas derived from mdx-iPS (DYS-HAC) cells, but not in those derived from mdx-iPS cells



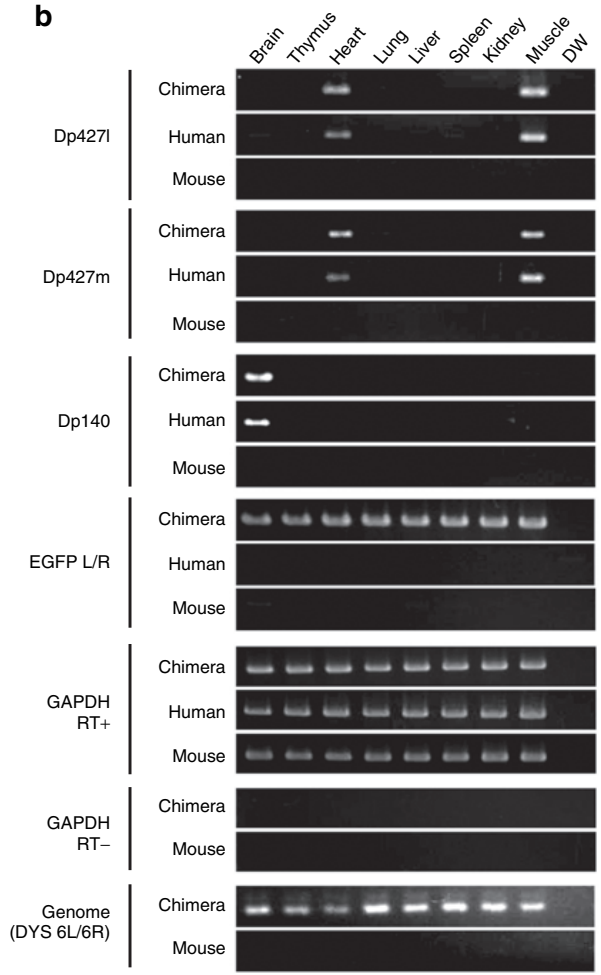


(e) Immunohistochemical analyses of dystrophin in muscle-like tissues of each teratoma. Immunodetection of mouse and human dystrophin (left panel), immunodetection of human-specific dystrophin (middle panel), and GFP micrography (right panel) are shown. The insets show enlarged images of immunohistochemistry. Nanog-iPS- and mdx-iPS- derived teratomas were used as positive and negative controls, respectively.

tissues from chimeric mice produced with mdx-iPS- DYS-HAC

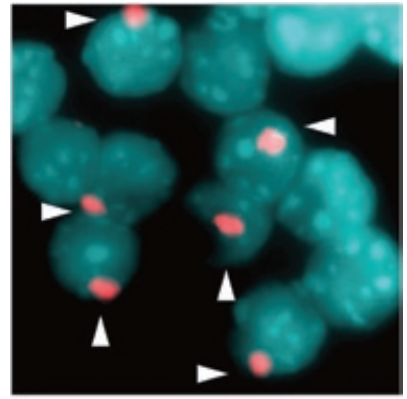


(a) Chimeric tissues derived from mdx-iPS (DYS-HAC). Bright (left panel) and fluorescence (right panel) micrographs are shown.



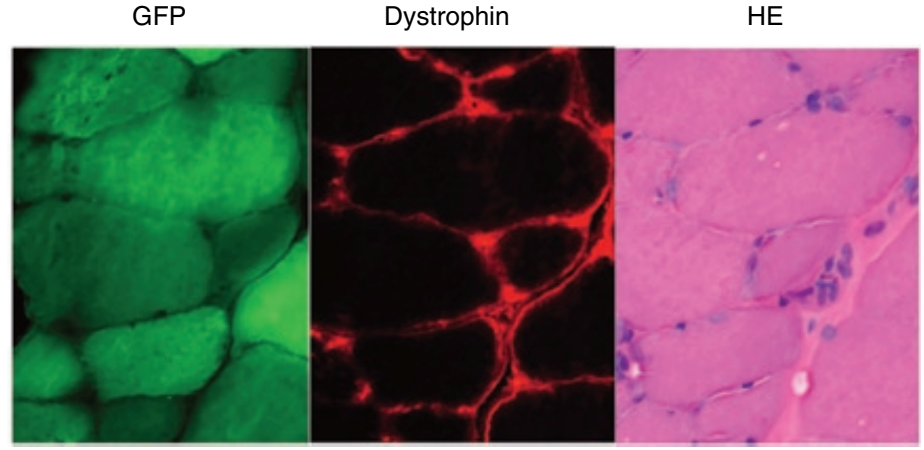
(b) Representative genomic PCR and RT-PCR data for detection of DYS-HAC in each chimeric tissue.

FISH analysis of chimeric kidney derived from mdx-iPS (DYS-HAC) cells.



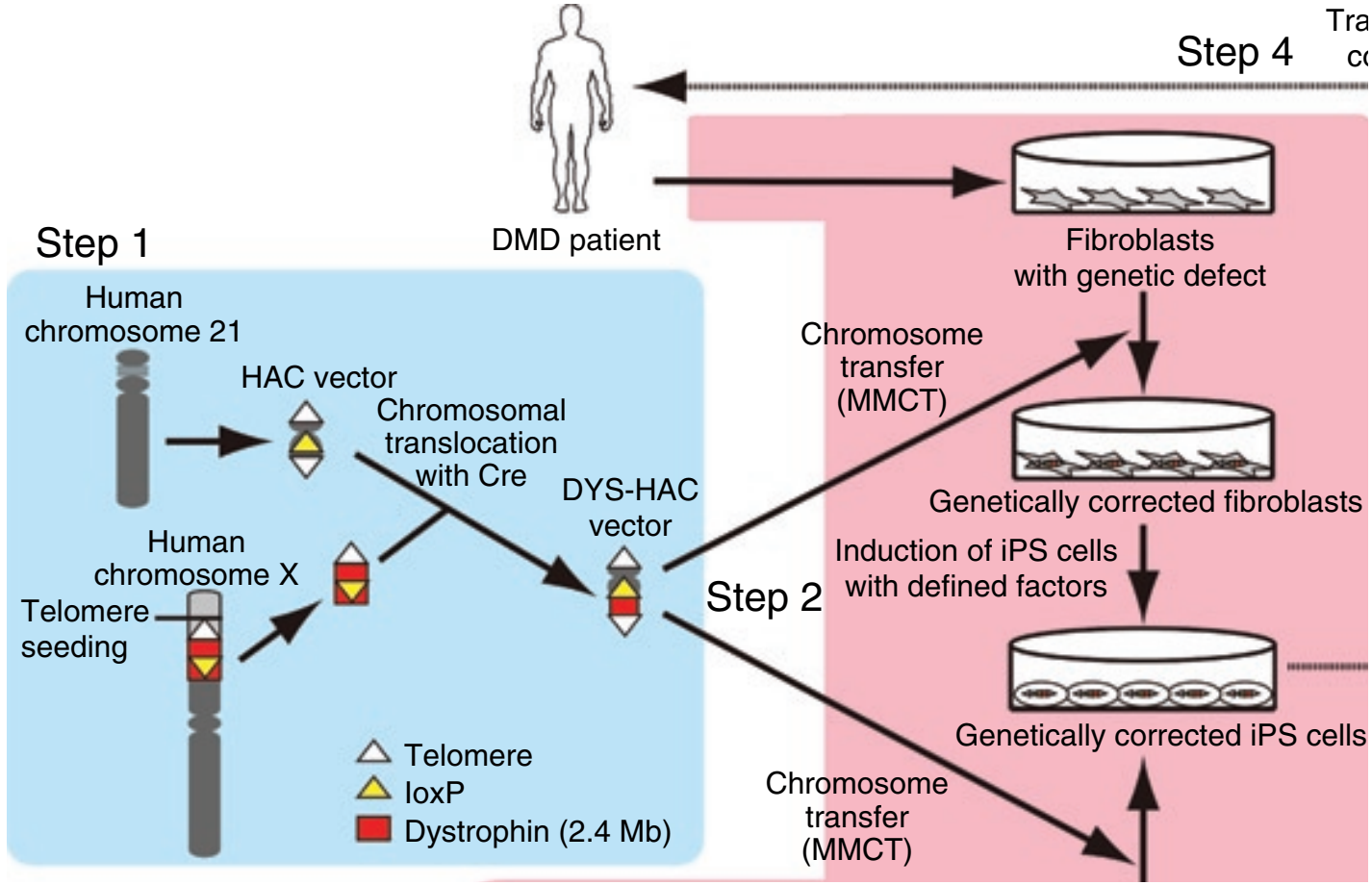
DYS-HAC (red); DNA (DAPI). Arrowheads show nuclei containing the DYS-HAC.

chimeric musclue



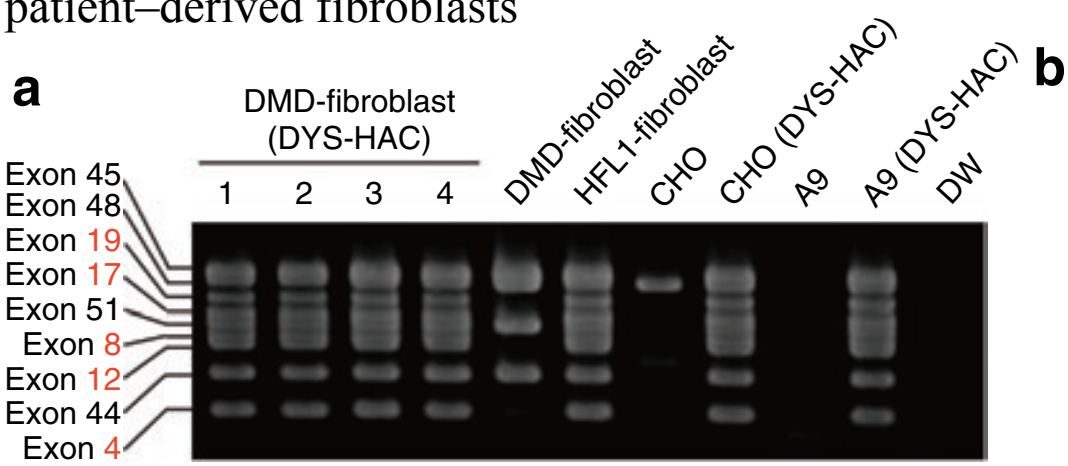
(d) Immunohistochemical analyses of dystrophin in chimeric muscle. HE staining (top panel), immunodetection of dystrophin (middle panel), and GFP micrography (bottom panel) are shown.

correction of DMD cells with DYS-HAC

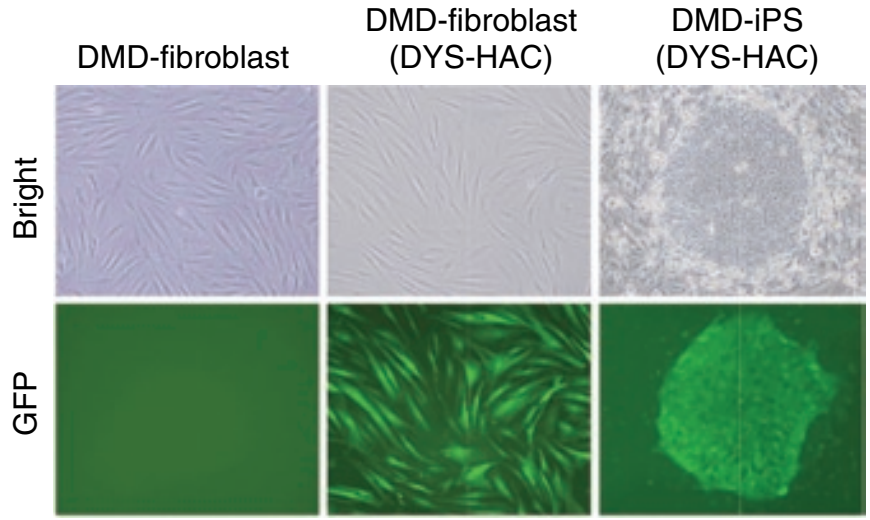


correction of DMD-fibrobalst cells with DYS-HAC

correction of iPS cells derived from a DMD patient DMD with deletion of exons 4–43.
A large deletion of this type cannot be corrected even using homologous recombination or other conventional vectors.
The DYS-HAC was transferred to DMD patient–derived fibroblasts via MMCT, as they failed to directly transfer the HAC into human iPS or human ES cells.
The DYS-HAC was successfully transferred to DMD-fibroblasts, as shown by PCR and multiplex PCR analyses
FISH analyses showed that the DYS-HAC was present as an individual chromosome in DMD patient–derived fibroblasts



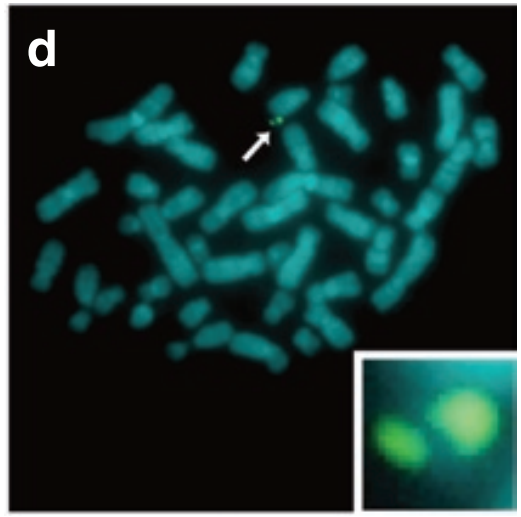
(a) Genomic multiplex PCR analyses for detecting the dystrophin genome on the DYS-HAC in DMD-fibroblast cells. Red lines show exons deleted in the DMD-fibroblast.



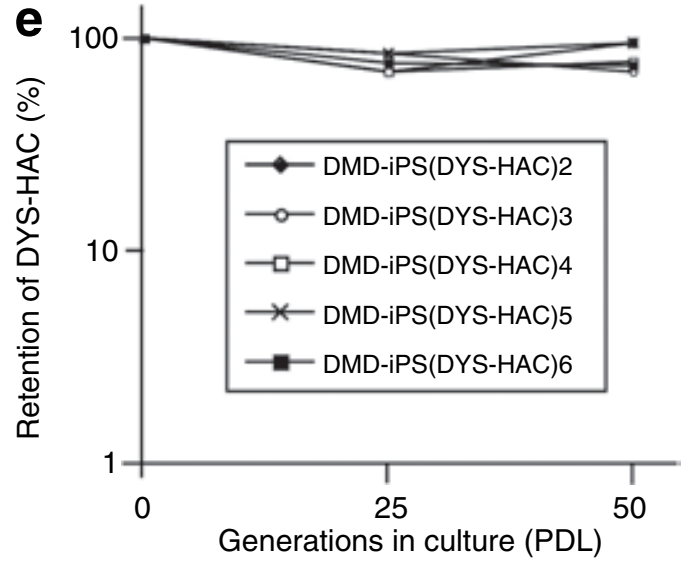
(b) Morphology of DMD-fibroblasts, DMD-fibroblasts (DYS-HAC), and DMD-iPS (DYS-HAC) cells. Phase-contrast (top panel) and fluorescence (bottom panel) micrographs are shown.

DMD-iPS (DYS-HAC)

The iPS cells were generated from the DMD-fibroblasts with the DYS-HAC using a combination of lentiviral infection with mouse Slc7a1 and retro- viral infection with KLF4, SOX2, OCT4, and c-MYC. PCR and multiplex PCR analyses showed that the DYS- HAC was maintained in all examined iPS cells, comparable to parent DMD-fibroblast (DYS-HAC) clones. FISH analyses showed that the DYS-HAC was present as an individual chromosome in the DMD-iPS (DYS- HAC) cells. iPS cells were cultured for about 4 months without selection. FISH analyses revealed that the DYS-HAC was independently and stably maintained in DMD-iPS (DYS-HAC) cells

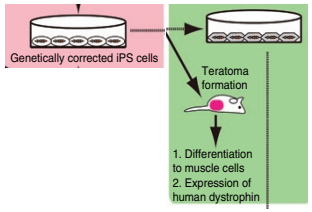


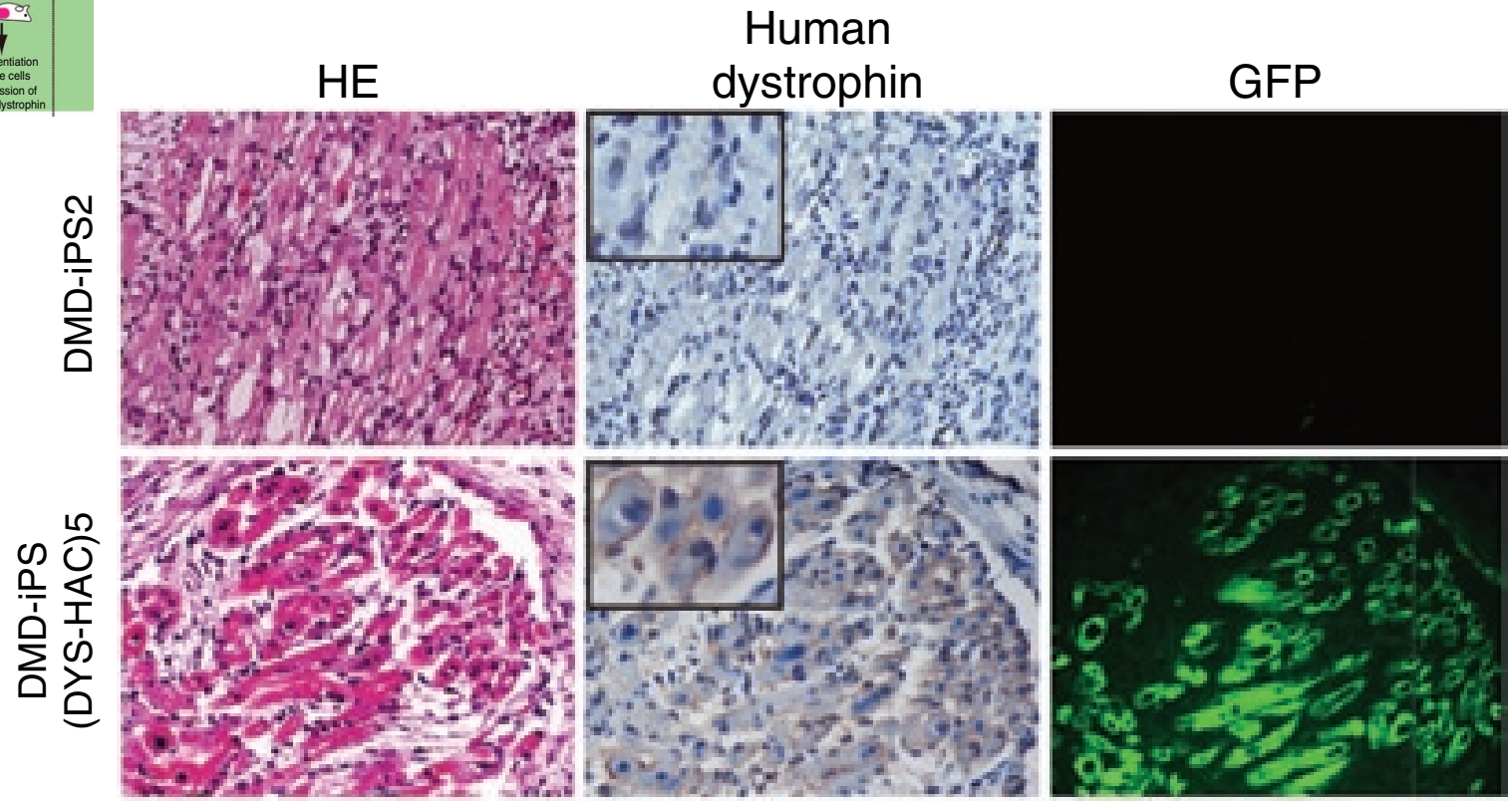
(d) FISH analyses for DMD-iPS (DYS-HAC) cells. An arrow indicates the DYS-HAC and the inset shows an enlarged image of the DYS-HAC.



(e) Mitotic stability of the DYS-HAC in DMD-iPS (DYS-HAC) cells.

human dystrophin in muscle-like tissues of teratomas derived from DMD-iPS (DYS-HAC)





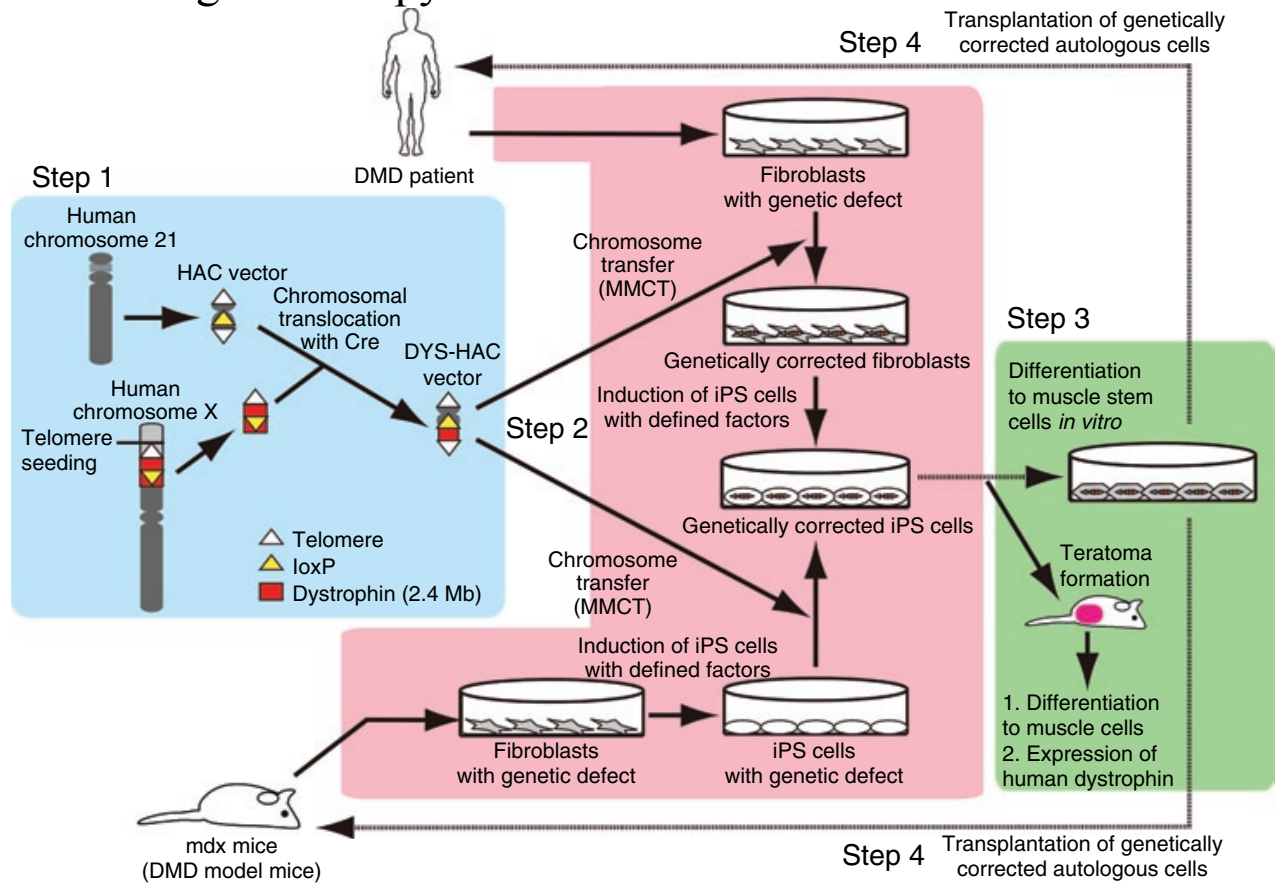
control iPS from DMD fibroblast

iPS from DMD –DYS-HAC

(f) Immunohistochemical analyses of dystrophin in muscle-like tissues of each teratoma. HE staining (left panel), immunodetection of dystrophin (middle panel), and GFP micrography (right panel) are shown. The insets show enlarged images of immunohistochemistry.

iPS cells combined with HAC vector system may open a way to more sophisticated DMD gene therapies.

Genetic correction of patient-specific iPS cells by MMCT of the DYS-HAC, efficient differentiation from iPS cells into muscle stem cells in vitro, and transplantation of genetically corrected autologous cells into the same patient are needed for the gene therapy of DMD.



consultare

Genomic Context Vectors and Artificial Chromosomes for Cystic Fibrosis Gene Therapy

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Kazuki and Oshimura. Human Artificial Chromosomes for Gene Delivery and the Development of Animal Models. ***Molecular Therapy (2011)*** vol. 19 no. 9, 1591–1601