De novo formed satellite DNA-based mammalian artificial chromosomes and their possible applications

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Abstract

Mammalian artificial chromosomes (MACs) are non-integrating, autonomously replicating natural chromosome-based vectors that may carry a vast amount of genetic material, which in turn enable potentially prolonged, safe, and regulated therapeutic transgene expression, and render MACs as attractive genetic vectors for "gene replacement" or for controlling differentiation pathways in target cells. Satellite DNA-based artificial chromosomes (SATACs) can be made by induced de novo chromosome formation in cells of different mammalian and plant species. These artificially generated accessory chromosomes are composed of predictable DNA sequences and they contain defined genetic information. SATACs have already passed a number of obstacles crucial to their further development as gene therapy vectors, including: large-scale purification; transfer of purified artificial chromosomes into different cells and embryos; generation of transgenic animals and germline transmission with purified SATACs; and the tissue-specific expression of a therapeutic gene from an artificial chromosome in the milk of transgenic animals. SATACs could be used in cell therapy protocols. For these methods the most versatile target cell would be one that was pluripotent and self-renewing to address multiple disease target cell types, thus making multilineage stem cells, such as

adult derived early progenitor cells and embryonic stem cells, as attractive universal host cells.

List of abbreviations

ACEs – Artificial Chromosome Expression system

CHO – Chinese Hamster Ovary

EB – Embryoid Body

EGFP – Enhanced Green Fluorescent Protein

ESC – Embryonic Stem Cell

EPO - Erythropoietin

FACS – Fluorescence Activated Cell Sorting

HAC – Human Artificial Chromosome

hESC – human Embryonic Stem Cell

HGH - Human Growth Hormone

HPRT - Hypoxanthine guanine PhosphoRibosyl Transferase

HSC - Hematopoietic Stem Cell

iPS – induced Pluripotent Stem

MAbs – Monoclonal Antibodies

MACs – Mammalian Artificial Chromosomes

MMCT - Microcell-Mediated Chromosome Transfer

MRI - Magnetic Resonance Imaging

MSC – Mesenchymal Stem Cell

PET - Positron Emission Tomography

SATAC - SATellite DNA-based Artificial Chromosome

Background on Mammalian Artificial Chromosomes (MACs)

Many groups worldwide have developed MACs and these technologies have been extensively reviewed (Ascenzioni et al. 1997; Basu and Willard 2005; Basu and Willard 2006; Bridger 2004; Brown et al. 2000; Cooke 2001; Csonka 2011; Duncan and Hadlaczky 2007; Grimes et al. 2002; Grimes and Monaco 2005); Hadlaczky 2001; Larin and Mejía 2002; Lewis 2001; Lim and Farr 2004; Lipps et al. 2003; Macnab and Whitehouse 2009; Monaco and Moralli 2006; Ren et al. 2006; Kouprina et al. 2013; Kouprina et al. 2014). Four different strategies have been developed for the construction of artificial chromosomes (Irvine et al. 2005): (i) in the synthetic approach the artificial chromosome is assembled from chromosomal components (Basu et al. 2005a; Basu et al. 2005b; Grimes et al. 2002; Harrington et al. 1997; Henning et al. 1999; Ikeno et al. 1998; Ikeno et al. 2009; Kaname et al. 2005; Kouprina et al. 2003; Nakashima et al. 2005; Suzuki et al. 2006), (ii) the 'top down' method applies the *in vivo* telomere-associated fragmentation of existing chromosomes (Au et al. 1999; Auriche et al. 2001; Carine et al. 1986; Choo et al. 2001; Farr et al. 1995; Heller et al. 1996; Ishida et al. 2000; Katoh et al. 2004; Mills et al. 1999; Shen et al. 2000; Voet et al. 2001; Wong et al. 2002) , (iii) naturally occurring minichromosomes may be engineered to construct an artificial chromosome (Raimondi 2011), and (iv) de novo chromosome generation can be induced via targeted amplification of specific chromosomal segments (Carl 2004; Csonka et al. 2000; Holló et al. 1996; Keresö et al. 1996; Lindenbaum et al. 2004; Praznovszky et al. 1991; Tubak et al. 1991). To date, two technologies, the 'top down' and the induced *de novo* chromosome generation approaches have advanced to the point suitable for biotechnological applications. The artificial chromosome technology exploits the unique properties of artificial chromosomes as being engineered chromosomes with defined genetic contents, capable of functioning as non-integrating vectors with large carrying capacity and stability (Duncan and Hadlaczky 2007; Hadlaczky 2001; Katona et al.

2011). The incorporation of different integrase and recombinase recognition sequences and integrase/recombinase enzyme genes into artificial chromosomes greatly improved the feasibility of integrating a transgene into the artificial chromosome (Duncan and Hadlaczky 2007; Katona et al. 2011; Kazuki and Oshimura 2011; Kouprina et al., 2013; Lindenbaum et al. 2004) . A MAC as a genetic vector should (1) possess large DNA payload capacity of 1 Mb or greater, (2) facilitate efficient gene(s) loading, (3) be capable of being transferred from one cell to another, and (4) enable stable, high-level transgene expression. Presently, there are three MAC technologies that have made the most progress in addressing these stringent requirements. First one is a class of truncated natural chromosomes (21ΔqHACs and 21ΔpqHACs), the second type is a *de novo* formed HAC (alphoid^{tetO}-HAC) and the third is the satellite DNA-based artificial chromosomes (SATACs and ACEs). By systematically deleting euchromatin from natural chromosomes, truncation-derived MACs preserve functioning centromeres, telomeres, and DNA origins of replication, while minimizing potential gene dosage effects in host cells. Of particular interests are the two MACs that were generated by telomere-directed truncation of the distal q ("long") arm of human acrocentric chromosome 21 generating 21ΔqHAC, and the subsequent truncation of the remaining distal p ("short") arm generating 21ΔpqHAC (Katoh et al. 2004; Kazuki et al. 2011; Oshimura and Katoh 2008) . Of note, the remaining p arm of 21ΔqHAC is assumed to encode only a rDNA gene tandem array and pericentromeric heterochromatin and hence should be genetically "neutral." Transgenes that have been efficiently loaded and expressed from these vectors include hypoxanthine guanine phosphoribosyl transferase (HPRT) (Ayabe et al. 2005; Grimes et al. 2001; Mejía et al., 2001) , enhanced green fluorescent protein (EGFP) (Yamada et al. 2006), antibody/cytokine receptor chimera (Kawahara et al. 2007), erythropoietin (EPO) (Kakeda et al. 2005), DNA-dependent protein kinase catalytic subunit (DNA-PKcs, critical for DNA repair) (Otsuki et al. 2005),

proinsulin (Suda et al. 2006), human dystrophin (Messina et al. 2009), CD40L (Yamada et al. 2008), the human P53 (Kazuki et al. 2008), telomerase (Oshimura et al. 2008), genes for human growth hormone (HGH), polycystic kidney disease (PKD1) and beta-globin (Basu et al. 2005a), CFTR (Auriche et al. 2002; Rocchi et al. 2010), GCH1 (Ikeno et al. 2002), factor IX (Breman et al. 2008), NBS1 and VHL genes (Kim et al. 2011) and STAT3 (Ikeno et al. 2009). Several of these genes were loaded as genomic copies carrying all the necessary control regions for proper wild type gene expression.

Overview of *de novo* formed stallite-based mammalian artificial chromosomes (SATAC)

This review focuses on SATAC technology. The *in vivo* generation of SATACs is based upon the induction of large-scale amplification via integration of exogenous DNA into specific chromosomal regions (Holló et al. 1996). The short arm of acrocentric chromosomes, which contains tandemly repeated ribosomal RNA genes (rDNA) and different non-coding satellite DNA sequences, is an optimal chromosomal region for inducing *de novo* chromosome formation. Basically, any cultured mammalian cell line that contains rDNA region is suitable for the production of *de novo* formed SATACs. Figure 1 describes a step by step overview of SATAC formation. The possibility to use any mammalian cell line for SATAC formation is a major advantage of this technology over other *de novo* MAC systems. SATACs and ACEs primarily consist of heterochromatin and pericentromeric sequences, which are purported to be devoid of gene-coding sequences except for rDNA (Csonka et al. 2000; Hadlaczky et al. 1991; Hadlaczky 2001; Holló et al. 1996; Keresö et al. 1996; Lindenbaum et al. 2004; Praznovszky et al. 1991). There is no evidence in humans that amplification of these pericentromeric sequences is deleterious to an individual, as polymorphisms in the short arms of acrocentric chromosomes have been shown to consist of amplified pericentromeric heterochromatin and/or rDNA (Conte et al. 1997; Friedrich et al. 1996;

Sands 1969; Sofuni et al. 1980; Stergianou et al. 1993; Trask et al. 1989A; Trask et al. 1989B; Verma et al. 1977; Warburton et al., 1976) and have been inherited with no adverse effects (Bernstein et al. 1981; Cooper and Hirschhorn 1962; Schmid et al. 1994; Therkelsen 1964; Yoder et al. 1974). Furthermore, supernumerary chromosomes derived from amplified pericentromeric heterochromatin and/or rDNA sequences of acrocentric chromosomes have been shown to be stably inherited through 1-3 generations in humans without any deleterious effects of phenotypes (Adhvaryu et al. 1998; Blennow et al. 1994; Brøndum-Nielsen and Mikkelsen 1995; Crolla et al. 1997; Hadlaczky et al. 2002; Howard-Peebles 1979; Mukerjee and Burdette 1966). Additionally, SATACs have been engineered with multiple (>50) site-specific recombination acceptor sites (*att*P) which enable the unidirectional loading of heterologous DNA (encoding an *att*B recombination donor site) *via* a mutant lambda integrase (Carl 2004; Lindenbaum et al. 2004) . The DNA recombination is unidirectional in mammalian cells, since the engineered integrase lacks the bacterial co-factors necessary for excision. The combination of the multiple *att*P sites on the ACEs and the unidirectional mutant integrase enables multiple loadings (during a single transfection) or sequential loadings (*via* multiple transfections) of transgenes (Kennard et al. 2009B; Lindenbaum et al. 2004). Transgenes that have been efficiently loaded and expressed from SATACs include enhanced green fluorescent protein (EGFP), red fluorescent protein (RFP), erythropoietin (EPO), monoclonal antibodies (MAbs) and therapeutic galactocerebrosidase transgenes to treat Krabbe disease in the Twitcher mice (Adriaansen et al. 2006; Carl 2004; Katona et al. 2008; Kennard et al. 2009A; Kennard et al. 2009B; Lindenbaum et al. 2004). In addition, specific MAb productivities of 55 pg/cell/day and 4 g/L yields in non-optimized bioreactors have been attained after transferring MAb-SATACs to various industrial strains of CHO cells, demonstrating high level SATAC-encoded transgene expression (Kennard et al. 2009a).

Isolation and transfer of SATACs

SATACs are purified to near homogeneity by high-speed flow sorting due to their unique nucleotide composition (a predominance of AT base pairs to GC base pairs compared with endogenous host cell chromosomes) (deJong et al. 1999), and are the only technology that permits the large scale production of purified gene-loaded MACs. Isolated SATACs have been microinjected into the pronuclei of murine embryos to generate transgenic mice (Blazso et al. 2011; Co et al. 2000) . On the other hand, very little success has been made in microinjecting mammalian cells *in vitro* due to the large outer diameter sizes (2.3 – 3.2 μm) of the microinjection needles (Monteith et al. 2004). The delivery of transgene-loaded SATACs to primary mammalian cells is a fundamental challenge for gene therapy applications. Microcell-mediated chromosome transfer (MMCT) has been the most commonly used technique for transferring MACs to various cell types. This method is tedious and generally inefficient (10^{-7} to 10^{-5}) (Doherty and Fisher 2003; Fournier and Ruddle 1977; Meaburn et al. 2005; Schor et al., 1975). Recently gene-loaded 21ΔqHACs have been transferred to human primary fibroblasts (Kakeda et al. 2005) and to human hematopoietic stem cells (HSCs) (Yamada et al. 2006) at clinically relevant frequencies of 1.26×10^{-4} and 4.0×10^{-4}, respectively. Nevertheless, despite these increased transfer efficiencies, during the process of microcell formation the host cell generates a heterogeneous population of microcells encapsulating endogenous chromosomes as well as MACs. Consequently, this transfer technique increases the probability that host chromosomes and chromosomal fragments will be co-transferred with MACs to the clinical target cells, which may result in unknown and potentially deleterious effects to the patient. It was also demonstrated that a mouse artificial chromosome vector developed from a natural mouse chromosome by chromosome engineering was more stably maintained in adult tissues and hematopoietic cells in mice

than HAC vectors. These MACs were stably maintained in human HT1080 cells in vitro during long-term culture, in mice at least to the F8 and F4 generations in ICR and C57BL/6 backgrounds, respectively, and in hematopoietic cells and tissues derived from old mice (Kazuki et al. 2013) . Since SATACs could be purified to near homogeneity, it was possible to transfer them to various cell types, including human mesenchymal stem cells (MSCs) (Vanderbyl et al. 2004) and HSCs (Vanderbyl et al. 2005) using commercially available cationic reagents and dendrimers (de Jong et al. 2001) with high transfer efficiencies (10^{-2}-10^{-4}). SATACs loaded with a therapeutic transgene have been transferred to murine ESCs by dendrimer-mediated chromosome transfer, establishing stem cell clones carrying intact SATACs, which was verified by fluorescent *in situ* hybridization (Katona et al. 2008; Katona, 2011). These murine ESCs were used to treat Krabbe disease in the Twitcher mice. The combination of flow cytometry purification and cationic reagents/dendrimer mediated delivery of SATACs into embryonic stem and iPS cells opens the road for cell therapy based applications. First of all, these cell types could be cultured and expanded indefinitely. Once the SATAC was delivered, proper cell lines could be selected and analyzed. We keep only those for further experiments, which carry an intact SATAC, express the therapeutic genes at the desired level and there is no trace of random integration and undesired chromosome fragments in the cells. These embryonic stem and iPS cells have high value for cell therapy, because these could be differentiated into the desired cell types. The differentiated cell types could be separated from undifferentiated ones by FACS. This is achieved by a fluorescent marker gene that is only active in the differentiated cell line and expressed from the SATAC. Sorted cells are used for direct cell therapy. Adult stem cells are also available for SATAC delivery as it was discussed earlier. These stem cells are multipotent and could be differentiated to various cell types. Embryonic and adult stem cell banking may decrease the costs of the technology. It was speculated that about 100 cell lines might be enough to give

immunocompetent cells for every patients in a given human population. So, in theory cell banking could be done for any particular disorder with the proper therapeutic SATAC included.

Stability of SATACs

In general MAC stability has been assessed in immortalized cell lines, such as human HT1080 fibrosarcoma cells, LMTK- and CHO rodent cells . The karyotypes of these cells vary greatly in culture, particularly after rounds of MMCT, cationic lipid/dendrimer transfections and drug selection, which in turn may not replicate the human primary cell environment for the MAC centromere function. The transfer of SATACs into primary human MSCs and HSCs demonstrated both autonomous chromosome maintenance and stable transgene expression (Vanderbyl et al. 2004; Vanderbyl et al. 2005). Mouse ES cell lines carrying therapeutic SATACs maintained high quality karyotypes, preserved their pluripotent state (which was verified by the expression of four widely accepted pluripotency markers alkaline-phosphatase, Nanog, Sox2 and Oct3/4), and expressed the therapeutic transgene (Katona et al. 2008). Under non-selective culture conditions, 98.9 % of the stem cells retained the therapeutic ACE through more than 100 cell generations. When the colonies of these cells were differentiated to embryoid bodies (EBs), therapeutic SATAC chromosomes were found in ~99 % of the nuclei of the EBs (Katona et al. 2008). These cells were differentiated into cell types of all three germ layers and SATACs were present in the nuclei of these cells (Katona et al., unpublished results). Transgenic animals have been generated by pronuclear microinjection of purified SATACs (Co et al. 2000), and by blastocyst injection of murine ESCs modified with SATACs (Katona et al. 2008). Additionally, SATACs have been passed on to more than three generations through the mouse germline demonstrating meiotic stability (Co et al. 2000), providing further evidence that SATAC centromeres function properly *in vivo*.

The SATAC transgenic founder mice and their progeny were healthy, robust, and free of neoplasms which provided initial evidence that SATACs are safe for *in vivo* applications (Co et al. 2000).

How safe is a MAC?

MACs are prospectively safer alternatives to viral gene therapy vectors, which have been shown to produce insertional oncogenesis (Cavazzana-Calvo et al. 2003; Morillon et al. 2003), be associated with chromosomal deletions/rearrangements (Miller et al. 2002; Miller et al. 2004), induce systemic toxicity (Christ et al. 1997), demonstrate shedding in patient semen (Couto 2004), , and cause serious antigenic responses (Evans et al. 2008; Raper et al. 2003). These safety issues may be addressed with non-integrating, autonomously replicating MACs, thereby providing mitotic stability and consistent vector/transgene copy number, as well as eliminating viral deleterious effects. As described earlier, SATACs are professedly devoid of gene sequences that could lead to indirect or direct dosage effects in cells destined for patient implantation. Moreover, transferring flow sorted SATACs by cationic/dendrimer reagents assures that only the therapeutic SATAC is delivered to patients, reducing the risk of delivering host chromosomes and chromosomal fragments *via* MMCT. SATACs also carry a suicide gene built-in. Presently, it is the thymidine kinase gene from *Herpes simplex* virus. A simple treatment with an orally administered drug (e.g. Aciclovir) can eliminate all the SATAC carrying cells from the patient's body. Certain therapeutic procedures, like cancer treatment, may even require this therapeutic cell elimination step after the treatment was successfully concluded. Recently, HAC vectors were produced with a new safeguard system and they can be easily eliminated from cells by inactivation of the HAC kinetochore via binding of tTS chromatin modifiers to its centromeric tetO sequences (Cardinale et al. 2009; Kononenko et al. 2013; Nakano et al. 2008) .

Combined mammalian artificial chromosome stem cell applications

The applications for stem cell therapy are as diverse as the scope of target diseases, therefore, the host stem cell with the most potential would be one with multi-lineage capacity, such as embryonic stem cells and adult derived early progenitor cells. Initially human embryonic stem cells (hESCs) derived from embryos caused ethical resistance. However, this issue seem to have been addressed by the successful establishment of permanent ESC lines (Guhr et al. 2006), the isolation of pluripotent spermatogonial stem cells from adult testis tissue (Guan et al. 2006) , and the generation of fully pluripotent stem cells from cultured human primordial germ cells (Donovan and de Miguel 2003; Shamblott et al. 1998), from single blastomere (Klimanskaya et al. 2006), individualized ESC cell lines through somatic cell nuclear transplantation (Hochedlinger and Jaenisch 2006). A novel option for deriving multi-lineage cells was the formation of induced pluripotent stem (iPS) cells generated from various human and mouse cell types by introducing protein and RNA factors and small molecules under ESC culture conditions (Inoue et al. 2014; Takahashi and Yamanaka 2006; Takahashi et al., 2007; Yamanaka 2012; Yu et al. 2007). Unfortunately, these iPS cells are different from hESCs in many respects such as epigenetic status and initial differentiation potential. However, we can consider the two cell types as complementary, interdependent biomedical research tools (Li et al. 2012; Orkin and Hochedlinger 2011; Papp and Plath 2013; Scott et al. 2011). Direct transplantation of ESCs and iPS cells can result in the development of teratomas (Evans and Kaufman 1981; Thomson et al. 1998) representing a serious safety issue. There is a controversy regarding the direct therapeutic application of MSCs, namely, that transplanted MSCs continue to replicate *in vivo* and preferentially incorporate into tumor stroma, constituting a significant fraction of the stromal tissue, possibly supporting tumor growth and induce resistance to chemotherapy (Aboody et al. 2006a; Aboody et al.

2006b; Danks et al. 2007; Houthuijzen et al. 2012; Hung et al. 2005; Karnoub et al. 2007; Koh and Kang 2012; Orimo et al. 2005; Roodhart et al. 2011; Studeny et al. 2004; Zhu et al. 2006). The risk of tumorigenesis may be overcome with the use of stem cells partially differentiated *ex vivo,* prior to transplantation. ESC differentiation models have established the potential to generate large numbers of lineage specific cells for cell replacement therapies. Mesoderm-derived lineages, including the hematopoietic lineages (Burt et al. 2004; Kondo et al. 2003; Kyba et al. 2002; Nakayama et al. 2000; Park et al. 2004), vascular (Bautch et al. 1996; Doetschman et al. 1985; Yamashita et al. 2000), and cardiac (Hidaka et al. 2003; Kolossov et al. 1998; Min et al. 2002; Min et al. 2003; Müller et al. 2000; Yang et al. 2002; Zandstra et al. 2003), are among the easiest to generate from ESCs and have been studied in considerable detail. Furthermore, methods have been established for selectively expanding multipotential hematopoietic cell populations (Kyba et al. 2003; Pinto et al. 1998), neutrophils (Lieber et al. 2004), megakaryocytes (Eto et al. 2002), mast cells (Tsai et al. 2000), eosinophils (Hamaguchi-Tsuru et al. 2004), dendritic cells (Fairchild et al. 2003), and erythroid cells (Carotta et al. 2004; Chang et al. 2006). Several studies have provided evidence for the generation of endoderm-derived cell types including populations that display characteristics of pancreatic islets (Colman 2004; D'Amour et al. 2005; D'Amour et al. 2006; Stoffel et al. 2004), hepatocytes (Hamazaki et al. 2001; Jones et al. 2002; Yamada et al. 2002a), thyrocytes (Lin et al. 2003), lung (Ali et al. 2002), and intestinal cells (Yamada et al. 2002b). Different protocols have also been established to promote neuroectoderm differentiation: oligodendrocytes capable of forming myelin sheaths around host neurons when transplanted into a myelin-deficient rat model of multiple sclerosis (Brustle et al. 1997), glial precursors (Brustle et al. 1999), midbrain dopaminergic neurons (Barberi et al. 2003; Kim et al. 2002), primitive neural stem cells (Tropepe et al. 2001). In addition to the neural lineages, ESCs can also give rise to epithelial cells that will undergo

differentiation to populations that express markers of keratinocytes (Bagutti et al. 1996). ES-cell-derived keratinocytes were able to form structures that resemble embryonic mouse skin, indicating that they possess some capacity to generate a functional tissue (Coraux et al. 2003). Retinal cells (Osakada et al. 2009) were also produced from human ES cells. Exhaustive studies will have to be conducted to determine whether induced ESC differentiation generates *bona fide* functional cells *in vivo*. The adult derived blastomere-like and epiblast-like stem cell types (Young et al. 2005) exemplifies prime candidates with ES-like differentiation capacity. Multipotent stem cells have been derived from bone marrow (Serafini et al. 2007; Young et al. 2005), dental pulp (Atari et al. 2011; Atari et al. 2012; Petrovic and Stefanovic 2009), adipose tissue (Fraser et al. 2006), and amniotic fluid (De Coppi et al. 2007). In addition, MACs encoding synthetic or natural genetic networks may be used to program pluripotent stem cells (natural or MAC-generated iPS cells) to follow specific lineage pathways to minimize the potential tumorigenicity issues.

Clinical Applications of MAC-modified stem cells

Pluripotent or multipotent stem cells carrying SATACs encoding diverse combinations of transgenes may be considered for a variety of clinical applications. These SATACs expressing complex gene pathways could be converted into "bioreactors" generating blood cells (e.g., platelets, red blood cells), immune cells (e.g., artificial antigen presenting cells), and pancreatic islet cells. Xenogeneic or allogeneic transplantable organs may someday be produced in which SATAC-encoded sequences could "down regulate" (by RNA interference technologies) the expression of host stem cell MHC class I/II loci and substitute MAC-encoded patient specific loci and appropriate
transgenes to eliminate the lifetime need for immunosuppressive drugs posttransplantation. Recent discoveries that stem/progenitor cells preferentially home to tumor sites (Aboody et al. 2006A; Aboody et al. 2006B; Danks et al. 2007; Studeny et al.

2004) offer the possibility for tumor therapy with artificial chromosomes armed with an arsenal of transgenes, including a drug-prodrug suicide system, cytokines (e.g., interferon-β) and antibodies.

Cell therapy

At present, there is no ongoing clinical trial with artificial chromosomes. However, preliminary results of preclinical experiments (Adriaansen et al. 2006; Auriche et al. 2001; Auriche et al. 2002; Carl 2004; Kakeda et al. 2005; Katona et al. 2008; Kawahara et al. 2007) have made a case for the feasibility of MAC-based *ex vivo* cell therapies where constitutive or overexpression of therapeutic gene(s) is acceptable or required. The successful transfer of MACs to MSCs (Bunnell et al. 2005; Ren et al. 2005; Vanderbyl et al. 2004), HSCs (Doherty and Fisher 2003; Vanderbyl et al. 2005) and ESCs (Katona et al. 2008), and the subsequent MAC transgene expression in differentiated cell types have opened a wide range of indications, including, hematological diseases, immunodeficiency diseases, lysosomal storage diseases and cancer (Bunnell et al. 2005; Carl 2004) . The non-exhaustive list above indicates the broadness of diseases and disorders which could be targeted with specific strategies and protocols (e.g., central nervous system, see review (Goldman 2005)). In most of the cases, generation of stem cell derived therapeutic lineages require multiple transgenes, including a combined positive/negative selection and/or marker system (Billon et al. 2002; Klug et al. 1996; Li et al. 1998), in order to eliminate non-differentiated stem cells. Platform ACEs by means of multiple acceptor sites have the capacity to harbor such complex genetic accessories. Recently, a novel method was described to load multiple genes onto the Platform ACEs by using only two selectable marker genes (Tóth et al. 2014). These markers may be removed from the ACEs before therapeutic application. This novel technology could revolutionize gene therapeutic protocols targeting the treatment of complex disorders and cancers. It could

also speed up cell therapy by allowing researchers to engineer a chromosome with a predetermined set of genetic factors to differentiate adult stem cells, embryonic stem cells and induced pluripotent stem (iPS) cells into cell types of therapeutic value. Most importantly these stem cells can potentially avoid immune response, because they may originate from the patient's own tissues. It is also a suitable tool for the investigation of complex biochemical pathways in basic science by producing a SATAC with several genes from a signal transduction pathway of interest.

Tracking and eliminating SATACs in patients

SATACs may be engineered to express an artificial magnetic resonance gene encoding lysine rich-protein (Gilad et al. 2007) or human protamine-1 (Bar-Shir et al. 2014). The location of the SATACs and any *in vivo* derived differentiated tissue may be detected by magnetic resonance imaging (MRI), providing a powerful non-invasive approach to monitor therapeutic cell fate. Micro-PET analysis also can be used to follow cell fate in vivo (Gyöngyösi et al. 2008). Finally, these therapies could be terminated by loading SATACs with the thymidine-kinase gene of *Herpes simplex* virus. An orally administered drug (e.g. Aciclovir) treatment may eliminate all the cells that are carrying the SATAC. Therefore, the large payload capacity of SATACs will ultimately enable reprogramming of pluripotent stem cells to specific lineages, therapeutic transgene expression, surveillance of tissue engraftment, and the precise termination of therapy.

Closing remarks

While the imperfection of viral vectors still hamper the long awaited breakthrough in gene therapy, mammalian artificial chromosomes are being developed as potentially safer genetic vectors for the therapeutic modification of human stem cells. HACs and SATACs have made the greatest progress in demonstrating efficient gene loading and the subsequent transfer and stable transgene expression in embryonic and adult stem cells.

Crucially these genetic vectors were maintained as autonomous chromosomes that facilitated transgene expression during stem cell differentiation and animal development. These proof-of-concept studies are the first steps towards exploiting their potential for safer and coordinated control of stem cell differentiation and therapeutic transgene expression. To bring MAC-based stem cell therapies to the clinic, further advances in MAC technology should be made, including the demonstration of inducible promoter systems required for the fine-tuned control of therapeutic transgene expression and the potential activation of suicide transgenes, like *Herplex simplex* virus thymidine-kinase, for managed gene therapy termination; the loading and expression of genetic pathways that control the lineage specific differentiation of pluripotent stem cells; and the establishment of MAC platform stem cell lines demonstrating controlled differentiation and long-term transgene expression and subsequent cell banking for better and faster therapies. MAC-modified stem cells have great potential to address a wide spectrum of diseases. Amongst these the most prominent are the therapies for genetic disorders without viable treatments where the constitutive overexpression of single or multiple transgenes are necessary and cancer therapies. The success of these trials will enable the engineering of genetic pathways and networks, which in turn will facilitate greater control of therapeutic transgene expression, tracking transplant fate, and transplant safety, thereby realizing the potential of modifying stem cells with mammalian artificial chromosomes for the treatment of human diseases.

Acknowledgments

I'm greatly indebted to the late Gyula Hadlaczky for the discovery and development of SATACs, and also for facilitating me to work, learn and progress scientifically under his kind supervision. Our work was supported by Hungarian National Technology Program Grant: *TECH_09_A1-DKMACTER* (http://www.nih.gov.hu/english).

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Figure legends

Figure 1. A step by step overview of SATAC formation. **A** The drawing shows the major components of an acrocentric chromosome that carries rDNA genes on the short arm. **B** A foreign DNA including the gene(s) of interest is integrated into the rDNA region of the acrocentric chromosome. Homologous rDNA sequences are either co-transfected or co-constructed with the foreign DNA to facilitate higher probability of specific integration. **C** Foreign DNA integration induces large scale, replication-dependend amplification in the short arm of these acrocentric chromosomes. This type of amplification process occurs in mammals and even in humans in nature with a low frequency. **D** A duplication of a functional centromere can take place in the amplification process. **E** A breakage occurs in the region between the two centromeres when cells divide. **F** An independent chromosome, the *de novo* SATAC is formed. This SATAC has all the necessary components for proper maintenance, replication and segregation in cell cycle and cell division. The SATAC also contains and expresses the integrated gene(s) of interest.