Cytologia focus:

Which Is a Reliable Approach in the Generation of Artificial Minichromosomes, Bottom-Up or Top-Down?

Satoru Fujimoto and Sachihiro Matsunaga*

Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science,
2641 Yamazaki, Noda, Chiba 278–8510, Japan

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Summary Artificial minichromosomes are exciting transgenic tools because they replicate and segregate autonomously and are not integrated into the host genome. They enable efficient delivery of an extremely large gene or multiple genes with high stability. There are two approaches to construct artificial minichromosomes: bottom-up and top-down. The construction of artificial minichromosomes addresses the basic questions of the structure and function of chromosomes. Advances in DNA synthesis technology will open a new era of generating artificial minichromosomes.

Key words Chromosome structure, Minichromosome, Artificial chromosome, Centromere, Telomere, Synthetic chromosome, DNA synthesis.

Cytogenetical and karyotypic studies have revealed variations of chromosomes in size, morphology, and number in various clades of plant and animal species (Al-Dakan and Al-Saleh 2015, Al-Qahtani *et al.* 2014, Arslan and Zima 2014, Bagane *et al.* 2014, Çakmak and Koca 2014, Deshpande *et al.* 2014, El-Esawi and Sammour 2014, Gholave *et al.* 2014, Haroun and Alotaibi 2015, Jantararat *et al.* 2014a, 2014b, Jumrusthanasan *et al.* 2015, Kaewmad *et al.* 2014, Kaewsri *et al.* 2014a, 2014b, 2014c, Kantek *et al.* 2015, Kasiroek *et al.* 2014, Kaur and Singhal 2015, Khakhong *et al.* 2014, Kumar *et al.* 2015, Kumar *et al.* 2014, Li *et al.* 2015, Maneechot *et al.* 2015, Martin *et al.* 2015, Miura and Iwatsubo 2014, Parai and Mukherjee 2014, Park 2015, Patawang *et al.* 2014a, 2014b, 2015, Phimphan *et al.* 2015, Pierozzi and Baroni 2014, Pinthong *et al.* 2014, 2015, Qumsiyeh *et al.* 2014, Rezaei *et al.* 2014, Samanta *et al.* 2015, Sato *et al.* 2015, Supanuam *et al.* 2015, Supiwong *et al.* 2015, Tahami *et al.* 2014, Tanomtong *et al.* 2014a, 2014b, 2015, Venkatesh and Munirajappa 2015, Venkatesh *et al.* 2014, Walia *et al.* 2015). Cytogenetical techniques including banding methods and FISH have revealed the evolutionary process in structural and number changes of chromosomes (Lombello and Pinto-maglio 2014, Mahbub *et al.* 2014, Manzum *et al.* 2014, Tungkajiwangkoon *et al.* 2015, Bansal and Kaur 2015, Akter *et al.* 2015, Hizume 2015, Hizume and Kan 2015, Imran *et al.* 2015, Kantek *et al.* 2015, Shibata and Hizume 2015, Matsunaga 2016). The chromosome evolutionary pro-

cess or the effect of chromosomal changes is speculative, and it is difficult to reproduce the process and mechanism. However, construction of artificial chromosomes can supply an answer to the basic question of how chromosomal structure forms (Nakano *et al.* 2008).

An artificial minichromosome can act as a new endogenous chromosome in the host cells. The artificial chromosomes are capable of replicating and segregating autonomously. Artificial chromosomes can be used for delivering an extremely large genetic region or multiple genes to host cells without integration into the host genome, thus preventing the position effect of introduced genes, insertional mutagenesis and genome instability (Costantini *et al.* 1989, al-Shawi *et al.* 1990, Reuter and Spierer 1992, Dobie *et al.* 1996, Yin *et al.* 2004, O'Connor and Crystal 2006). There are two strategies for producing artificial chromosomes (Hadlaczy 2011, Gaeta and Krishnaswamy 2011) (Fig. 1). The first one involves the *de novo* assembly of artificial chromosomes with artificial compositions of cloned chromosomal components delivered into cells (bottom-up approach). The second one involves truncation of endogenous chromosomes to create small chromosomes (top-down approach).

The bottom-up approach was first established in yeast (Murray and Szostak 1983). The primary components of a yeast artificial chromosome are the autonomously replicating sequence, a centromere, and telomeres from the yeast. Vectors larger than 55 kb with the three components became relatively stable through mitosis and meiosis (Murray and Szostak 1983). In mammalian cells, the bottom-up approach to generate mamma-

* Corresponding author, e-mail: sachi@rs.tus.ac.jp
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Fig. 1. Generation strategy of artificial minichromosomes: bottom-up approach *versus* top-down approach. ARS: autonomously replicating sequence, CEN: centromere sequence, TEL: telomere sequence.

lian artificial chromosomes (MACs) was successfully adapted by using synthetic arrays of centromeric alpha satellite DNA with telomeric DNA in human HT1080 cells (Harrington *et al.* 1997, Ikeno *et al.* 1998). The resulting linear microchromosomes, containing foreign alpha satellite DNA, were mitotically stable and bound centromere proteins. Moreover, telomeric sequences were not essential for *de novo* chromosome formation as a circular MAC from the alpha satellite DNA (Ebersole *et al.* 2000). However, during the delivery to the cells, the DNA was subjected to multimierization resulting in artificial chromosomes which are much larger than the original constructs used for the transformation. Moreover, reproducible generation of MACs has only been achieved in the HT1080 cell line (Irvine *et al.* 2005). In plant cells, the bottom-up approach was also attempted. Only maize cells with bottom-up artificial chromosomes were reported using centromeric sequences (Carlson *et al.* 2007, Ananiev *et al.* 2009). After biolistic bombardment of maize centromeric sequences into embryonic maize tissue and subsequent transgene selection, microscopic analysis revealed some small fragments with centromeric signals independent of the host maize chromosomes. These fragments were reported to be transmitted during mitosis and meiosis. However, information about whether the fragments have centromere proteins or not is lacking (Carlson *et al.* 2007), and the effect of telomere mediated chromosome truncation cannot be denied (Ananiev *et al.* 2009). So, *de novo* assembly of autonomous maize minichromosomes remains a matter of debate (Houben *et al.* 2008, Birchler *et al.* 2010).

Although the bottom-up approach has been successful in budding yeast and human HT1080 cells, many cells often only exhibited inserted centromeric sequences without centromeric activity (Baum *et al.* 2006, Phan *et al.* 2007). An idea arises that the epigenetic state of the centromere may be important rather than the primary DNA sequence (Karpen and Allshire 1997). One attempt is made using *Drosophila melanogaster*. It has been described with the *lacO*/LacI system (Hirakawa and Matsunaga 2016), which is based on the artificial targeting of the LacI-tagged CENP-A of *Drosophila* (CID) to transgenic tandem repeat arrays of the *lacO* sequences (Mendiburo *et al.* 2011). Surprisingly, the tethering of

the fusion protein CID-GFP-LacI alone is sufficient not only for the ectopic centromere formation, but also for the establishment of extrachromosomal *lacO* minichromosomes, which are mitotically stable for several cell generations, even after eliminating CID-GFP-LacI (Mendiburo *et al.* 2011). To establish an epigenetically stable state for the CENP-A assembly, the *tetO*/*tetR* system was used (Ohzeki *et al.* 2012). The *tetO*/*tetR* system is based on the artificial targeting of the *tetR*-tagged histone modification enzyme to *tetO* sites within the synthetic alpha satellite DNA arrays. The exogenous histone acetyltransferase activity is required for the kinetochore assembly on the extrachromosomal *tetO* containing array. The kinetochores no longer require this exogenous activity to maintain their structure and function (Ohzeki *et al.* 2012).

The top-down approach is accomplished by truncating the natural chromosome by several different routes. In fission yeast, minichromosome Ch¹⁶ and its derivatives were created by γ -irradiation (Niwa *et al.* 1986). The minichromosomes were derived from the pericentromeric region of chromosome III and from 120 to 750 kb in length (Niwa *et al.* 1989). To generate such a kind of truncated minichromosomes, the double strand breakages should occur at multiple sites (*e.g.*, near centromere and telomere), so the efficiency is quite low. Introduction of cloned telomeric repeats into mammalian cultured cells cause chromosome breakages by formation of new telomere at the integration site (Farr *et al.* 1991). This technique was further refined for generation of MACs (Farr *et al.* 1992, Itzhaki *et al.* 1992) including using homologous recombination-proficient chicken DT40 cells *via* microcell-mediated chromosome transfer (Kuroiwa *et al.* 1998). The telomere mediated approach was also used for generation of truncated chromosomes in various organism including *Candida albicans* (Kabir and Rustchenko 2005) and *Xenopus* (Wuebbles and Jones 2007). In plants, maize (Yu *et al.* 2006), *Arabidopsis thaliana* (Nelson *et al.* 2011, Teo *et al.* 2011), barley (Kapusi *et al.* 2012), and rice (Xu *et al.* 2012) were reported. However, for the minichromosome generation in plants, further breakthroughs like DT40 cell mediated truncation in mammalian cells are required because of the inefficiency of target integration of telomeric sequence.

A ring minichromosome was artificially generated by using the DNA sequence specific recombination system, Cre/*LoxP* combination with DNA transposon system, and activator (*Ac*)/dissociation (*Ds*) in *A. thaliana* (Murata *et al.* 2013). T-DNA was inserted into the centromere of chromosome 2 that contained two *LoxP* sites for Cre-mediated recombination. One of the *LoxP* sites was transposition by the *Ac/Ds* system to two *LoxP* sites containing centromeric region. Expression of the Cre recombinase excises the region between these *LoxP* sites, resulting in the formation of a ring minichromosome (Murata *et al.* 2013). In mammalian cells, 2.4 Mb of the gene can be successfully integrated into the MACs via Cre/*LoxP* site specific recombination system (Katoh *et al.* 2004, Ayabe *et al.* 2005, Hoshiya *et al.* 2009), and this system can be applied not only to cultured cells but also to mice (Kazuki *et al.* 2008). In plants, targeted transgene integration into minichromosomes also was achieved by using the Cre/*LoxP* system (Yu *et al.* 2007).

Advances in DNA synthesis technology may put an end to the conflict in the two approaches of the construction of artificial minichromosomes. Recently, a 273 kb-chromosome of *Saccharomyces cerevisiae* was completely synthesized (Annaluru *et al.* 2014). It is functional although its DNA sequence excludes subtelomeric regions, introns, tRNAs and transposons from native chromosome III. This success indicates that artificial minichromosomes were freely designed using the bottom-up approach to at least the ~1 Mbp level. In the near future, various types of artificial minichromosomes with desired functions and DNA sequences for animal and plant cells will be constructed using DNA synthesis technology.

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