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▶ To cite this version:

Daniele Fachinetti, Hiroshi Masumoto, Natalay Kouprina. Artificial chromosomes. Experimental Cell Research, Elsevier, 2020, 396 (1), pp.112302. 10.1016/j.yexcr.2020.112302 . hal-03452714

HAL Id: hal-03452714

https://hal-cnrs.archives-ouvertes.fr/hal-03452714

Submitted on 29 Nov 2021

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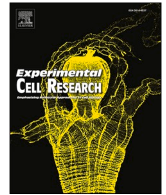
Contents lists available at ScienceDirect
Experimental Cell Research
journal homepage: www.elsevier.com/locate/yexcr


Artificial chromosomes

1. Introduction

Forty years have gone by since Clark & Carbon [1] cloned the first functional eukaryotic centromere DNA from budding yeast. A short centromere DNA segment, which was later limited to 125 bp, that showed classical Mendelian segregation when ligated into the Autonomous Replicating Sequence (ARS) plasmid. This discovery led to the development of Yeast Artificial Chromosomes (YAC), with the hopes of generating artificial chromosomes in mammals. However, in many animals and plants, including humans, centromeres occupy large regions mainly composed of repetitive DNAs (satellite DNAs). It took over 17 years to overcome the difficulties in analyzing and cloning such repetitive DNA, before two groups (Willard's and Masumoto's teams) successfully developed the first human/mammalian artificial chromosome (HAC/MAC) [2,3].

Artificial chromosomes (ACs) were considered as a promising system for functional gene delivery into mammalian cells as they are efficiently inherited both during mitosis and meiosis as nonessential additional chromosomes. ACs range in size from 0.5 to 10 Mb and can be fully engineered. ACs overcome many problems inherent to viral-based gene transfer systems such as limited cloning capacity, lack of copy number control, and insertional mutagenesis due to integration into host chromosomes that plague viral vectors. The ability of ACs to carry entire genomic loci with all regulatory elements allows them to faithfully mimic the normal pattern of natural gene expression. Now, after more than 20 years since their first development, HACs/MACs have greatly contributed to the field as large multi-gene loading vectors. They have also been used for various fundamental studies including chromosome instability (CIN) assays, along with playing a crucial role for the development of recombination and chromosomal transfer technology.

From the perspective of expanding synthetic genome research, artificial chromosomes and de novo centromere technologies are indispensable, but further development is necessary. This special issue "Artificial chromosomes" contains invited topical review articles from experts in the field of centromeres, neocentromeres, genome analysis, artificial chromosomes and synthetic genome in order to evoke thorough discussion.

Unlike budding yeast, in higher animals and plants the formation and maintenance of functional centromere and kinetochore structures is not a simple 1:1 correlation with the centromeric DNA. Epigenetic chromatin assembly mechanisms greatly influence the functional structures. The neocentromere phenomenon, by which a functional centromere/kinetochore structure is assembled and maintained in a rearranged chromosomal arm region uncoupled from the native centromeric DNA, is rather regarded as evolutionarily important. Moreover, even in humans the function(s) of centromeric repetitive DNA has/have not yet been fully elucidated. **Paul Talbert** and **Steve Henikoff** [4] provide a comprehensive overview of the different types of existing centromeres, from the point centromeres in yeasts to more complex regional monocentric and holocentric centromeres in multicellular organisms. The authors focus on the diversity of the centromeric DNA sequences, the DNA-depending features of eukaryotic centromeres and how/why such repetitive regions were generated during evolution. They finally discuss how these repetitive regions can form non-B-form DNA structures such as hairpins and loops and how they impact centromere biology, from transcription to nucleosome positioning, to centromere establishment.

Human centromeres, as well as those of many animals and plants, are located in large, highly repetitive satellite DNA regions. This posed limits for the complete assembly of the actual sequences. **Karen H. Miga** [5] focuses on the recent advances using long-read sequencing technologies and a variety of assembly methods to achieve this ultimate goal of complete chromosome assemblies in humans, the "telomere-to-telomere (T2T)". She introduces examples of the ultra-long read assemblies applied to the human X and Y centromeric alpha satellite arrays and discusses the development of computational platforms for a faster and automated ways to improve sequencing data assemblies.

Lori L. Sullivan and **Beth A. Sullivan** [6] provide an in-depth overview of human alpha satellite arrays and their dynamic properties. Specifically, the authors discuss several features of these megabase regions such as the structure of higher order repeats, the variations in array length and in monomer sequence and the stability of the different arrays. They also highlight the epigenomic variation of the human centromeres, from transcription and their produced RNA to DNA methylation. Finally, they describe the existence of centromeric epialleles, in which the two homologous chromosomes display centromere function at different high order repeats.

Riccardo Gamba and **Daniele Fachinetti** [7] review current knowledge of CENP-B – the only sequence specific centromeric DNA binding protein – and of its DNA binding site CENP-B box, combining phylogenetic and functional perspectives. They discuss the evolutionary history of CENP-B and describe the appearance and conservation of CENP-B box along phylogenesis. They report possible mechanisms of regulation of CENP-B activity and they propose how CENP-B may impact on DNA secondary structure, kinetochore stabilization and epigenetic control of centromere identity. They finally describe recent advances on the functional role of CENP-B in chromosome segregation both in mitosis and meiosis, with implications on the selective forces leading to the evolution of this protein.

Catherine Naughton and **Nick Gilbert** [8] summarize recent progress on neocentromere research from different species, including

https://doi.org/10.1016/j.yexcr.2020.112302

evolutionary new centromeres. Specifically, the authors focus on the organization and role of different types of chromatin found at neocentromeres; by describing the epigenetic status of centrochromatin and the surrounding chromatin, including their 3D organization, the authors highlight how heterochromatin contributes to the maintenance and functionality of centromeres. Overall, the studies of these repetitve-less centromeres provide relevant information for canonical centromeres.

The importance of the neocentromere as a model system to interrogate canonical human centromere is further discussed by **Marina Murillo-Pineda** and **Lars E.T. Jansen** [9]. Here, the authors summarize 40 years of history on centromere research from genetic elements of the point yeast centromeres to chromatin features of mammalian neocentromeres. They highlight in detail the importance of the histone H3 variant CENP-A and other epigenetic modifications in the maintenance of centromere identity in different species and their mechanism of action. Finally, focusing on studies of existing neocentromeres and artificial systems for neocentromere generation, they discuss how centromere specification is constantly drifting from genetic to epigenetic driven mechanisms.

Tetsuya Hori and **Tatsuo Fukagawa** [10] review the principal literature related to de novo centromere formation and the use of artificial centromeres as a model system to study the genetic and epigenetic regulation of such phenomenon. They outline the origin of neocentromeres, and what we have learned from both naturally occurring neocentromeres and experimentally induced ones. They further perform an in-depth comparison of DNA features and epigenetic marks at native centromeres versus the non-canonical centromeres. Finally, they highlight key features that impact on centromere biology such as regulation of centromere length and movement, a phenomenon defined as centromere drift, and discuss the use of artificial kinetochores to dissect their molecular mechanisms.

Yan Yu Wong and colleagues [11] provide an overview on the assembly and regulation of centromeres mainly at the point centromere of budding yeast and the *C. elegans* holocentric one (i.e., a particular type of centromere organization that spans along the entire chromosome). They discuss how centromeres are epigenetically regulated via histone modifications and variants, the underlying DNA features and the role of non-coding RNAs and transcription in the establishment and maintenance of centromere function both at endogenous sites and on artificial chromosomes. The authors finally discuss perspectives on the use of yeast and *C. elegans* artificial chromosomes to advance our understanding of centromere biology.

CENP-B binding to CENP-B boxes is necessary for de novo centromere and HAC formation, but, in contrast, it is not required for the maintenance of established centromeres and it is absent from the Y centromere or ectopically formed neocentromeres. Therefore, the function of CENP-B in chromosome segregation itself is still controversial. **Jun-Ichirou Ohzeki and co-authors** [12] introduce recent progresses in various HACs formation, including synthetic satellite DNAs and fusion-protein tethering systems and discuss how centromere chromatin and heterochromatin assemble on the introduced DNA in cells. They emphasize the importance of epigenetic chromatin assembly balance and maintenance mechanisms, focusing on the unique properties of CENP-B.

Epigenetic chromatin assembly and maintenance mechanisms greatly influences the functional centromere structures in many eukaryotes. **Craig W Gambogi and colleagues** [13] introduce the role of the genetic and epigenetic contributions to establish centromere identity, highlighting their recent work where they hijack the epigenetic machinery to initiate centromere identity on a new generation of HACs built without α-satellite DNA. By seeding the key epigenetic centromere marker CENP-A (the centromere-specific histone H3 variant) on the introduced DNA, HAC formation can be freed from technical difficulties of handling a long repetitive DNA. They also discuss the challenges in developing useful unique sequence-based HACs.

The international synthetic yeast genome project-Sc2.0 exemplifies how a classical synthetic biology "design-build-test-learn" engineering cycle can effectively test hypotheses about various genome fundamentals. **Zhouqing Luo and co-authors** [14] introduce this ambitious project to design and build synthetic eukaryotic yeast chromosomes, which is nearing completion. They describe the design principles and applications of Sc2.0 in a wide range of discussion. It is also an important framework for constructing complete synthetic human artificial chromosomes. The authors finally emphasize that the genome reshuffling SCRaMbLE system implemented in synthetic yeast strains provides unprecedented diversified resources for genotype-phenotype study and yeast metabolic engineering.

Masashi Ikeno and **Yoshinori Hasegawa** [15] describe applications of HACs in cell research and cell engineering. Because the HACs are mitotically and meiotically stable in mice, it is possible to investigate the tissue-specific expression of the genes inserted into the HACs. As example, these authors discuss chimeric mice harboring HACs that are stably transmitted to progeny from both males and females. The authors highlight that the built-in functional genes fall under the control of cell regulation. Thus, the HACs can reproduce the development of cell-specific and tissue-specific expression of the genes in transgenic mice. Such HAC-carrying mice have the potential to be a powerful tool for analyzing genes function.

Chromosome Instability (CIN) manifests unequal chromosome distribution during cell division and is a distinguishing feature of most cancer types. While CIN drives tumorigenesis, there is a threshold level beyond which CIN becomes a barrier to tumor growth. Therefore, CIN can be exploited therapeutically for cancer treatment with the identification of drugs that greatly elevate chromosome instability in cancer cells. **Kouprina and colleagues** [16] describe applications of HAC-based assays for identification of drugs that elevate (CIN) in cancer cells. The authors also describe a recent work that uses fluorescence HACs to identify compounds that specifically target telomeres/telomerase or to uncover novel human CIN genes that control mitotic progression with the usage of siRNA libraries. Finally, the authors describe how the identification of such compounds and novel CIN genes could lay the foundation for new treatment strategies for cancer.

One of the potential applications of the HAC-based vectors is in the field of gene therapy that aims at curing various human diseases. So far, the vast majority of gene therapy clinical trials have exploited viral vectors such as retroviral, lentiviral, adenoviral, and adeno-associated virus-based vectors. However, these gene delivery approaches have several fundamental limitations such as low cloning capacity, being able to accommodate only cDNA that cannot recapitulate the physiological regulation of the "damaged" endogenous loci, and ability to randomly integrate into the human genome. Artificial chromosome-based gene delivery vectors are episomal, non-integrative, featuring large size inserts containing native gene loci up to few Mb in size. **Daniela Moralli** and **Zoia L. Monaco** [17] discuss in detail advantages and challenges of ACs for gene transfer and gene therapy. They discuss the usage of herpes virus simplex type 1 (HSV-1) amplicons to transfer large fragments of DNA (up to 150 kb of exogenous DNA) containing alphoid DNA to human cells for HAC formation. The authors further highlight recent works that aim to increase the capacity of gene delivery.

MAC/HAC vector systems can be a suitable platform for chromosome-scale synthetic DNA assembly of such a huge DNA. In order to take advantage of artificial chromosomes maximally, it is also important to develop the chromosome transfer technology between cells from different organisms (for example, between yeast and human). **David M. Brown** and **John I. Glass** [18] describe how yeast and mammalian artificial chromosomes are developed and utilized, especially in the chromosome transfer technology and application. They discuss potential applications of MAC/HAC vectors constructed in the budding yeast cells using transformational-associated recombination (TAR) cloning and further yeast/human cells fusion.

Microcell-mediated chromosome transfer (MMCT) technology enables individual mammalian chromosomes, Mb-size chromosome

fragments or mammalian artificial chromosomes (MACs) to be transferred from donor to recipient cells (as Chinese hamster ovary and mouse A9 cells). In the past few decades, MMCT has been applied to various studies, including gene mapping, analysis of chromosome status such as aneuploidy, and in epigenetics. In this issue, **Suzuki and co-authors** [19] summarize the principles of MMCT MACs transfer and recent advances in the chromosome transfer technology. Specifically, they discuss methods for microcell–cell fusion to increase chromosome transfer efficiency. Finally, they discuss a recently established cryopreservation method to store microcells at −80 °C that significantly aids the MMCT protocol.

The humanized animals are invaluable for biomedical research because they can recapitulate specific human expression profiles across different species. In this issue, **Moriwaki and co-authors** [20] describe the history of transgenic animal development and a key role of ACs for the creation of the transgenic models for human disease, drug development, and humanized animal research. Specifically, they summarize works on the production of human antibodies by transchromosomic animals and the mouse models of human aneuploidy syndromes such as Down syndrome. In addition, the authors describe an application of humanized mice and rats developed using ACs for studies of drug metabolism, pharmacokinetics, and toxicity.

Sinenko and co-authors [21] describe a recent breakthrough and challenges in HAC technology in terms of its implementation for iPSC/ESC-based gene therapy and regenerative medicine. In particular, the authors discuss current achievements in developing a HAC-based technology for mouse gene therapy with the perspectives for gene replacement models of several monogenic human diseases. They highlight a recent progress from Oshimura's group in murine models of Duchenne muscular dystrophy (DMD) disease through the replacement of the affected dystrophin gene with the use of the top-down generated HAC technology.

The development of plant artificial chromosome technology is not only expected to answer questions in fundamental biology, such as the structure and function of plant genomes, but also to improve the yield and quality of plants, and to contribute to the efficient production of industrial materials and pharmaceuticals. However, due to technical barriers associated with plant transformation, centromere design, and meiotic transmission, the development of this technology is still far from being complete. **Kelly Dawe** [22] introduces animal artificial chromosomes and properly addresses such key concerns and steps to upcoming challenges in the design of fully synthetic plant centromere and chromosome.

In contrast to the situation in yeasts and humans, synthetic artificial chromosomes are not available for plants yet. **James A. Birchler** and **Nathan C. Swyers** [23] describe engineered minichromosomes that have been produced by telomere mediated chromosomal truncation, the only method successfully applied to reduce the size of naturally available chromosomes in few plant species. They describe truncation of the nonvital supernumerary B chromosome of maize as a favorite target, but engineered minichromosomes derived from the normal A chromosomes have also been recovered. They also discuss potential solutions to address the problem that, while clearly behaving normally for mitotic transmission, minichromosomes are lost during meiosis.

Acknowledgements

The editors would like to thank Bill Earnshaw (University of Edinburgh, UK) for promoting this special issue. D.F. receives salary support from the CNRS. H.M. was supported in part by MEXT KAKENHI Japan (grant number 20H00473) and the Kazusa DNA Research Institute Foundation.

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