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Review Article

Centromere identity and function put to use: construction and transfer of mammalian artificial chromosomes to animal models

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Mammalian artificial chromosomes (MACs) are widely used as gene expression vectors and have various advantages over conventional expression vectors. We review and discuss breakthroughs in MAC construction, initiation of functional centromeres allowing their faithful inheritance, and transfer from cell culture to animal model systems. These advances have contributed to advancements in synthetic biology, biomedical research, and applications in industry and in the clinic.

Introduction

Mammalian artificial chromosomes (MACs), including human and mouse artificial chromosomes, are built either from isolated [1] or synthetic [2–4] DNA. Once they are functional in cells, they essentially behave as small chromosomes that are carried alongside the natural chromosomes, either in cell culture or model animal systems. Their inheritance through cell divisions is conferred by the formation of a functional centromere, which is the chromosomal locus that directs the formation of the proteinaceous kinetochore that connects to the spindle microtubules that drive chromosome segregation to daughter cells [5] (note that other aspects of centromeres and kinetochores are the topics of the Essays in this issue). Since the first MACs were reported [6,7], they have held promise to advance synthetic biology applications with potential use in chromosome research and industrial/biomedical applications.

A simple MAC application is for the delivery of potentially useful genes to cells and organisms [reviewed in detail [8]. Unlike other approaches (e.g. viral delivery systems or 'stable transfection'), they do not require integration (or any other disruption) to the natural chromosomes of the host cell/animal. Further, they can carry large genes, replete with natural regulatory elements. This is due to essentially unlimited gene packaging size of MACs. This same property also permits the engineering of multiple genes into a single MAC.

MACs also provide potential tools for more radical genetic engineering projects. For instance, following on the successes of the Human Genome Project-Read [9,10] and the construction of synthetic yeast artificial chromosomes (YACs) [4], Human Genome Project-Write [11] was proposed. A central goal of that project is to construct an entire set of synthetic human chromosomes. Such an accomplishment would further the understanding of genetic blueprints (i.e., chromosome structure, gene regulation, genetic diseases, and evolution) and inspire new tools with which to attack diverse human health challenges. Designer genomes could create cells immune to viral infection or resistant to chemotherapeutics. Short of an entire set of human chromosomes, synthetic chromosomes could be used to deliver large sets of genes required for growing transplantable patient-personalized organs in pigs or other attractive donor species.

Synthetic eukaryotic chromosome technology has advanced furthest in budding yeast, including entirely synthetic yeast chromosomes [4,12]. One of the reasons these advances have occurred in this

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system is that budding yeast centromeres are small (125 bp), defined sequences [13]. Indeed, functional YACs have been major tools in molecular biology for more than 30 years [14]. Despite the progress in synthesis of YACs, there are significant obstacles toward applying these strategies to the synthesis of MACs, notably including the initial formation of a functional centromere. In mammals, centromere specification requires an essential epigenetic mark provided by nucleosomes containing CENP-A, a histone H3 variant [5,15]. Thus, there has been a challenge for the field to make the initial acquisition of specialized centromeric chromatin occur with sufficient efficiency to permit rapid isolation of cells carrying functional MACs. Moreover, MACs have been traditionally built primarily from 40–200 kb segments of the highly repetitive DNA found at centromere locations in natural chromosomes [1,6,7]. The large stretches of highly repetitive DNA are technically difficult to clone and amplify, and they compromise the recombination-based assembly schemes pioneered for the synthetic budding yeast cloning efforts [12]. Strategies that bypass the requirement for highly repetitive centromere DNA are therefore needed, and indeed, it is possible to do so by initially seeding non-repetitive centromere assembly sites with CENP-A nucleosomes [16]. Further innovations promise to bring MACs ever closer to realizing their potential, including advances with nascent centromere formation. Thus, further investigation of the organization and dynamics of mammalian centromeres promises to positively impact the advancement of MACs technologies. The introduction of MACs into animal models presents another set of unique challenges, which have thus far been addressed using an approach termed microcell-mediated chromosome transfer (MMCT) to move functional MACs into embryonic stem (ES) cells [17]. Our essay summarizes and discusses progress in MAC construction, establishment, transfer into ES cells, and introduction into animals.

Approaches for constructing MACs with functional centromeres

The two successful routes to isolating a functional MAC are 'bottom-up' and 'top-down': the *de novo* formation of a functional centromere containing the epigenetic CENP-A nucleosome mark, or using an existing natural centromere after removing the natural chromosome arms, respectively.

In landmark studies on the bottom-up construction of first-generation MACs [6,7], human α-satellite DNA was seeded together with telomeric DNA, or delivered in a cloned YAC constructs, in the human fibrosarcoma cell line HT1080. MACs (10–20% the size of natural human chromosomes) that had acquired functional centromeres formed and were mitotically stable for up to 6 months in culture without selection. These MACs were divided into categories based on different mechanisms of formation. In one category containing *de novo* centromeres, transfection of α-satellite DNA led to a telomere-directed truncation event following integration of telomeric DNA into an endogenous chromosome. In this case, the MACs were composed of genomic sequences from truncated endogenous chromosomes and centromeric DNA (human α-satellite) from transfection. Notably, acquired genomic sequences facilitated *de novo* centromere formation [6]. MACs in a second category containing *de novo* centromeres comprised transfected centromeric DNA, either in the context of YAC constructs or not, but lacked endogenously derived genomic sequences (Figure 1). Several variations on this general approach have been reported since these initial reports [2,18–24]. A general feature of bottom-up MACs is that the input DNA is multimerized at some point prior to functional MAC identification. This leaves the mechanism of multimerization up to 'post-mortem' analysis. Two universal rules for bottom-up MACs are the requirement for α-satellite DNA containing a high density of CENP-B boxes and the CENP-B protein [1,3,25]. These requirements are only for the centromere initiation step, and they can be completely bypassed by seeding CENP-A nucleosomes onto MAC template DNA (via initial tethering of the CENP-A nucleosome assembly factor, HJURP, using the bacterial Lac operator/repressor system) immediately upon introduction to cells [16,26]. Bottom-up MACs are typically formed in the human HT1080 cancer cell line. Other cell lines, such as HeLa, are thought to be more resistant because of a higher propensity to form heterochromatin (defined by the histone H3K9me3 modification) on incoming MAC DNA. This can be counteracted by artificial targeting of histone acetyltransferases to the MAC DNA, allowing functional MACs to form in HeLa, U2OS, and ES cells [27].

The top-down approach for MACs avoids the requirement for the *de novo* formation of a functional centromere by instead truncating and editing a natural human chromosome [17,28]. Early success with this strategy involved transfer of a natural mammalian chromosome to a homologous recombination-proficient chicken B cell line, DT40, where it can go through several rounds of 'stripping' via telomere-directed truncation [29]. The truncated chromosome can then be retrofitted with useful elements, such as antibiotic resistance genes (for selection in MMCT steps, discussed, below) and a loxP site adjacent to the functional centromere for downstream transgene insertion. Following these steps, the MACs can be transferred to mammalian cells. CHO cells are a common intermediate step due to their efficiency as microcell donors in the MMCT steps required to transfer into desired recipient cell lines [30] (Figure 2). To date, MACs have been generated by the top-down approach from human chromosomes 14, 21, X, and Y and

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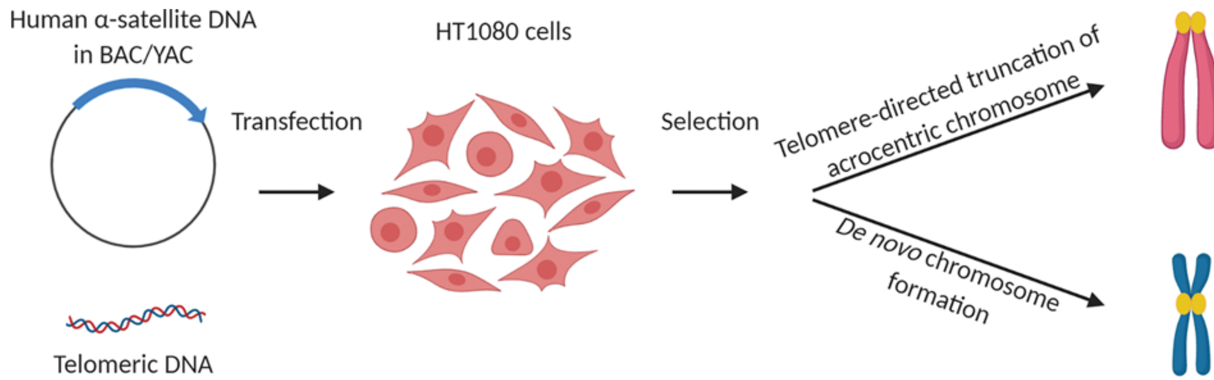

Figure 1. First-generation bottom-up approach for MAC construction

(*De novo* centromeres in yellow) Synthetic human α-satellite DNA was transferred together with telomeric DNA into the human fibrosarcoma cell line HT1080. The first category of MAC formed resulting from telomere-directed truncation of an endogenous chromosome following integration of telomeric DNA. MACs in a second category containing *de novo* centromeres comprised transfected centromeric DNA but lacked endogenously derived genomic sequences, which were barely detectable after DAPI staining even at high magnification (size difference not reflected in this schematic representation).

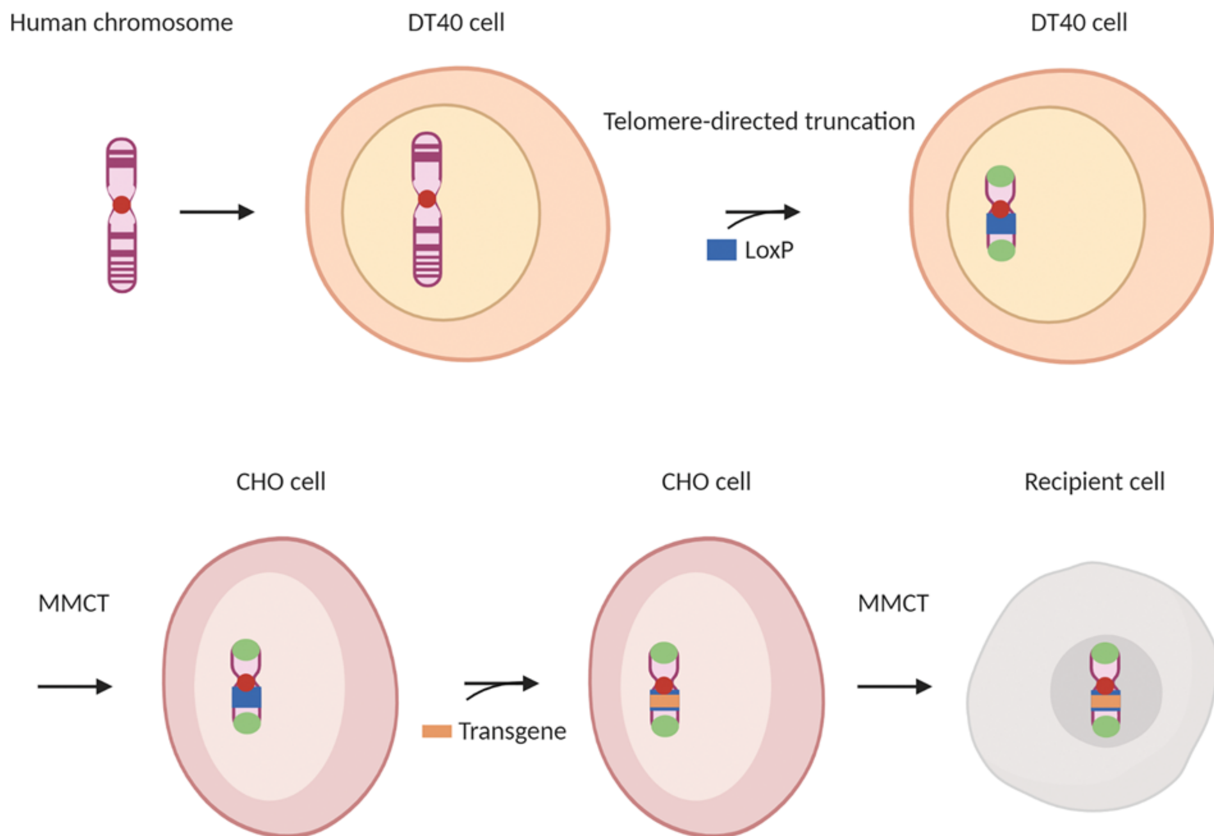

Figure 2. Top-down approach for MAC construction

A human chromosome was transferred to chicken DT40 cells. After several rounds of telomere-directed truncation of the human chromosome, a LoxP site was introduced for transgene insertion. Then the MAC containing a natural centromere (red), telomeres (green), and a LoxP site (blue) was transferred to CHO cells by MMCT. Next, a transgene (orange) was inserted into the LoxP site. After selection, the resulting MAC was transferred into a recipient cell.

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range from 0.5 to 10 Mb in size [28,29,31–35]. Depending on downstream applications, one potential disadvantage of MACs generated by the top-down approach is that some endogenous genes are retained even after several rounds of telomere-directed truncation [17,28]. MACs engineered from human chromosome 21 have been reported to carry no endogenous genes, however, eliminating this particular issue [34].

Technique to transfer functional MACs from one cell line to another

As alluded to, above, there are many instances where it is necessary to transfer MACs to intermediate or ultimate recipient cell types. MMCT [36] was originally developed for transferring an entire chromosome from donor cells to recipient cells, and has become a primary approach for MAC transfer [28,34,37–39]. A typical MMCT approach first involves prolonged mitotic arrest using microtubule poisons, leading many cell types to exit mitosis with chromosomes forming many micronuclei, rather than a single daughter nucleus. The micronuclei are then isolated by lysing cells and separating their contents through sedimentation in the centrifuge followed by filtration through 3-μm membranes to enrich for small membrane-enclosed microcells that contain one or a few chromosomes. The microcells are then used in subsequent membrane fusion steps, historically using surfactants like polyethylene glycol (PEG), with the intermediate or ultimate recipient cells.

The fusion step is limiting, and essentially all aspects of the MMCT protocol must be optimized for donor/recipient cell line pairs [30]. In some cases, PEG-mediated MMCT fusion steps can be optimized to the extent that sufficiently efficient MAC transfer can be achieved [40]. In other cases, PEG proves to be toxic to one or both lines, or the efficiency of MMCT is as low as one in a million cells [41]. In these cases, viral cell fusion approaches, which are based on viral-derived surface receptors that facilitate fusion, have been explored. In one instance, a measles virus envelope protein-mediated MMCT (MV-MMCT) method increased the efficiency 50- and 100-fold when recipient cells are HT1080 and human immortalized mesenchymal stem cells, respectively [41]. In MV-MMCT, donor CHO cells containing MACs are transfected with plasmids encoding the MV-fusion and hemagglutinin proteins. The resulting microcells can fuse with recipient cells that naturally express a cell-surface receptor, CD46, that recognizes MV. A limitation, therefore, is that the utility of MV-MMCT is restricted to fusions where there are high cell-surface levels of CD46 on recipient cells. In another instance of viral cell fusion approaches to MMCT, a method using murine leukemia virus (MLV), termed retro-MMCT, is applicable to various types of recipient cells derived from different primate and rodent species [42]. In retro-MMCT, the microcell donor CHO cells express envelope proteins derived from MLV as the fusogen. Depending on donor/recipient pairs, the benefits of viral MMCT approaches can be large. For instance, the efficiency of MAC transfer into NIH3T3 recipient cells is 26.5-times higher when using retro-MMCT rather than conventional MMCT [42]. Exploring diverse fusion approaches has led to many examples where MMCT has become a powerful strategy for the transfer of MACs into cell lines (e.g. ES cells, HT1080, K562, Caco-2, HepG2) [17,41,43,44] and for essential steps in the generation of animal (mouse) models, as discussed below.

Animal models carrying MACs

Generating animals carrying MACs builds on earlier success with generating animals containing extra chromosome fragments. Specifically, human chromosomes or chromosome fragments from human fibroblasts had been introduced into mouse ES cells using MMCT [45]. The ES cells were injected into host embryos to generate chimeric mice. In adult chimeric mice, the transferred human chromosomes or chromosome fragments were stably maintained, and human genes were expressed in a tissue-specific manner. Further, a fragment of human chromosome 2 (hChr. 2) was transmitted to the offspring through the germline. This study [45] demonstrated the possibility of delivering large exogenous DNA (a chromosome or chromosome fragment) into a mouse model (Figure 3).

The first chimeric mice carrying functional MACs were generated using a similar strategy [17]. Using a top-down MAC approach, a 10-Mb human chromosome region including the immunoglobulin λ-light chain (Igλ) element was engineered into a MAC construct in DT40 cells. It was transferred to CHO cells and then into recipient mouse ES cells that were injected into mouse eight-cell embryos. The MAC was stably maintained in the chimeric mice, and the Igλ gene in the MAC was expressed *in vivo*. The creation of this transchromosomic (Tc) mouse model carrying a MAC provides evidence for the utility of this system for *in vivo* expression and analysis of Mb-sized regions of human chromosomes. Subsequent similar strategies with MACs have produced Tc mouse models of human disease [46,47]. For example, Hsa21 Tc mouse strains contain an extra human chromosome 21 and display behavioral impairment and cardiac abnormalities, modeling aspects of Down syndrome patients [47]. Derivatives of this MAC can be used, in principle, to narrow down the genetic features/causative genes on human chromosome 21 that generate specific Down syndrome phenotypes.

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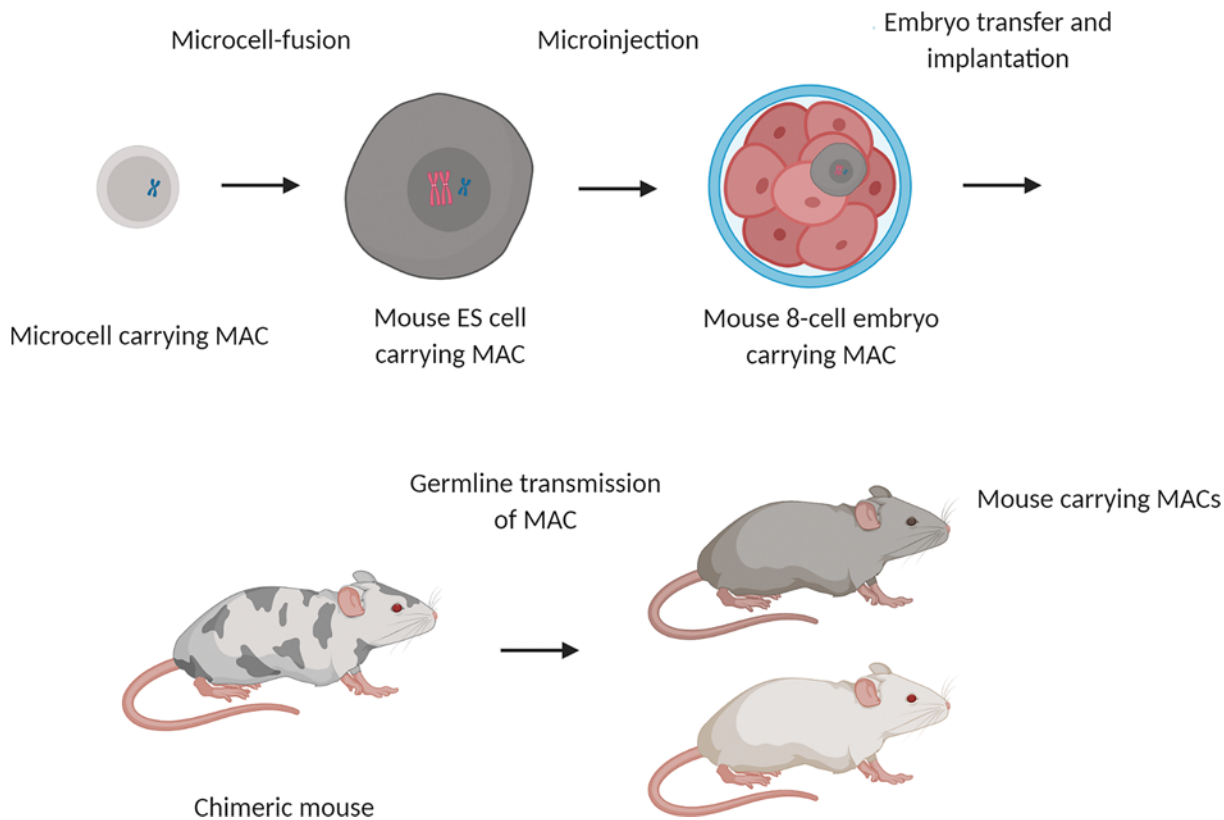

Figure 3. Scheme showing the progression from a microcell carrying a MAC to a chimeric mouse expressing human genes on the transferred MAC

By microcell-fusion, a microcell containing an engineered MAC (blue) fuses with a mouse ES cell (endogenous chromosomes in pink). Then the mouse ES cell carrying the MAC is microinjected into an eight-cell embryo. After embryo transfer and implantation, chimeric mice are obtained. If the MAC is present in the germline of the chimeric animal, it can be transferred to offspring.

MAC strategies are generally transferrable in diverse mammals. For instance, in bovine experiments, Tc calves have been generated that express human immunoglobulin heavy-chain and λ light-chain genes [38]. A MAC carrying the chromosome regions of interest was transferred to bovine primary fetal fibroblasts via MMCT. Tc fibroblast nuclei were transferred by cell fusion to enucleated bovine oocytes, which were used to generate embryos *in vitro*. After culturing to the blastocyst stage, the embryos were implanted into pseudo-pregnant cows. Tc fetuses were retrieved at less than 2 months of gestation to re-establish Tc fibroblast cell lines, which were genotyped and used for another round of nuclear transfer, embryo formation, and implantation to produce Tc calves. Finally, four offspring Tc calves expressing a repertoire of human immunoglobulin transcripts were born. The creation of such Tc animals is crucial for the development of systems capable of producing human polyclonal antibodies. More recently, an optimized Tc bovine system produced high yields of human polyclonal antibodies [48]. The Tc calves are potential producers of 'humanized' antibodies, enzymes, and various other proteins for therapeutic use.

The Tc mice and calves, mentioned above [38,46–48], are humanized animal models, since they carry and express functional human genes. Such models include tissue-humanized animals and genetically humanized animals and are widely used in synthetic biology [38,48], in the generation of human disease models [46,47], and in pharmacokinetic and toxicokinetic research [8]. A general necessity for generating humanized models has become clear as researchers realize that differences between species [and differences *in vitro* (i.e. cell culture models) versus *in vivo* (i.e. in animals)] impact the results of biomedical research and its applications. The need for accurate models of human biology has many profound real-world examples. A very well-known example is the use of thalidomide to treat hyperemesis gravidarum in pregnant women. Thalidomide use caused malformations in approximately 10 000 children worldwide before it was withdrawn at the beginning of the 1960s, despite the absence of fetal teratogenicity in previous tests in pregnant mice [49]. To improve this type of animal modeling, a humanized CYP3A mouse was generated by transferring MACs carrying a human CYP3A cluster, which encodes enzymes responsible for the

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oxidative metabolism of multiple therapeutic drugs, into a Cyp3a knockout mouse [50]. Thalidomide treatment of cultured whole embryos caused limb abnormalities in CYP3AMAC-positive mouse embryos, while it had no effect on CYP3AMAC-negative embryos, showing that humanized CYP3A mice carrying MACs are useful for drug toxicity tests. The MAC approach should be generalizable to future efforts to 'humanize' mouse models for drug development.

Outlook

The generation of various humanized animal models carrying MACs, both rodents [8] and calves [38,48], opens the door for even more ambitious goals for medicine and synthetic biology. Transformative advances await, such as transplantable human organs harvested from livestock animals [11]. In the nearer future, MACs will be expanded in coming years by building on the fact that they are capable of carrying multiple genes [51], features that will be crucial for generating powerful animal models for drug development. Combinatorial use of MAC and genome-editing technologies (e.g. CRISPR-based tools) [52–55] could generate SNP modifications in animal models recapitulating human subpopulations and is potentially useful for patient-specific drug discovery.

While most MAC-containing animals, to date, were generated using top-down MAC strategies, bottom-up approaches will likely emerge as an attractive alternative. Bottom-up construction, in principle, permits the ultimate synthetic control to generate completely designer chromosomes. In addition, rapid centromere acquisition may permit MAC formation in diverse cell types, bypassing the need for any cell transfer (i.e. the MMCT typically used in the MAC field) steps. The 'centromere problem' created by the requirement for epigenetic information to initiate formation of a functional MAC is being effectively addressed by hijacking the cellular machinery that propagates centromere identity on natural chromosomes [16]. These strategies are predicted to be broadly applicable in mammals (and beyond in many eukaryotes) because of the conserved nature of this cellular machinery [5,15].

In summary, progress in MAC construction and transfer technologies has already enabled the generation of humanized mouse models carrying MACs for investigating complicated biological and biomedical problems *in vivo*. In the future, MAC approaches are poised to efficiently generate humanized animal models and carry other useful synthetic gene payloads for utility across a broad range of applications in medicine and beyond.

Summary

- MACs are important tools for synthetic biology, biomedical research, and clinical applications.
- Two main methods to build MACs are bottom-up and top-down. Bottom-up approaches require the *de novo* formation of a functional centromere, while the top-down approach isolates an existing centromere from a natural chromosome away from its arms.
- MMCT is the technology widely used to transfer MACs between cell types and has represented an obligatory step preceding the generation of animal models carrying MACs.
- Mouse and other mammalian models carrying MACs have great potential in producing human proteins (enzymes, antibodies etc.) for research and therapeutic use, generating new animal models of human disease, and synthetic biology applications.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

All authors wrote and edited the manuscript.

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Abbreviations

ES cell, embryonic stem cell; GOI, gene of interest; Igλ, immunoglobulin λ-light chain; MAC, mammalian artificial chromosome; MLV, murine leukemia virus; MMCT, microcell-mediated chromosome transfer; MV-MMCT, measles virus envelope protein-mediated MMCT; PEG, polyethylene glycol; Tc, transchromosomic; YAC, yeast artificial chromosome.

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