Mammalian Synthetic Biology

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Mammalian Synthetic Biology: Time for Big MACs

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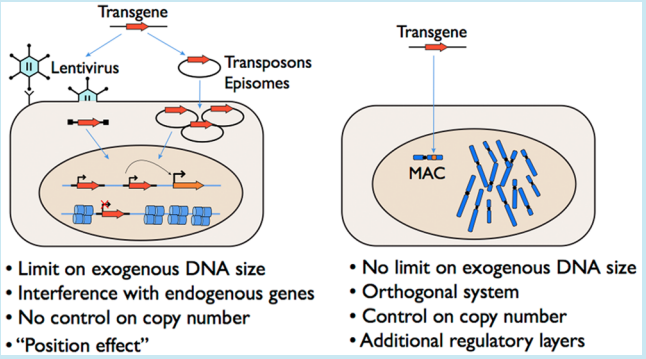
ABSTRACT: The enabling technologies of synthetic biology are opening up new opportunities for engineering and enhancement of mammalian cells. This will stimulate diverse applications in many life science sectors such as regenerative medicine, development of biosensing cell lines, therapeutic protein production, and generation of new synthetic genetic regulatory circuits. Harnessing the full potential of these new engineering-based approaches requires the design and assembly of large DNA constructs—potentially up to chromosome scale—and the effective delivery of these large DNA payloads to the host cell. Random integration of large transgenes, encoding therapeutic proteins or genetic circuits into host chromosomes, has several drawbacks such as risks of insertional mutagenesis, lack of control over transgene copy-number and position-specific effects; these can compromise the intended functioning of genetic circuits. The development of a system orthogonal to the endogenous genome is therefore beneficial. Mammalian artificial chromosomes (MACs) are functional, add-on chromosomal elements, which behave as normal chromosomes—being replicating and portioned to daughter cells at each cell division. They are deployed as useful gene expression vectors as they remain independent from the host genome. MACs are maintained as a single-copy and can accommodate multiple gene expression cassettes of, in theory, unlimited DNA size (MACs up to 10 megabases have been constructed). MACs therefore enabled control over ectopic gene expression and represent an excellent platform to rapidly prototype and characterize novel synthetic gene circuits without recourse to engineering the host genome. This review describes the obstacles synthetic biologists face when working with mammalian systems and how the development of improved MACs can overcome these—particularly given the spectacular advances in DNA synthesis and assembly that are fuelling this research area.



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Synthetic biology is an emerging area of research whose main aim is the design, construction and characterization of synthetic genetic circuits that once integrated into the cellular host produce desirable outputs in a robust and predictable manner.[1−3] In recent years, synthetic biology applied to mammalian cells has rapidly evolved and achieved impressive advancements and proof-of-principle studies for the generation of complex and diverse genetic devices.[1−3] Several functional genetic modules such as toggle switches,[4] boolean logic gates,[5] hysteretic switches,[6] oscillators[7] and light induced optogenetic switches[8] have been successfully developed in mammalian cells. It is now essential to generate more complex and therapy-oriented gene circuits, assembling modules into larger scale systems that are robust and predictable. This essential step will help synthetic biologists design and generate genetic circuits resembling the intriguing complexities of the native mammalian gene regulatory networks, allowing higher and finer grained of control over the cell behavior.

In this review, we will first discuss the issues of scalability, orthogonality and predictability that must be addressed for the implementation of more complex synthetic genetic circuits in mammalian systems. We go on to outline and discuss how the exploitation of mammalian artificial chromosomes (MACs) as delivery vehicles for transgenes and circuits might help tackle some of these issues.

CHALLENGES OF SYNTHETIC BIOLOGY IN MAMMALIAN SYSTEMS

Genetic engineering has traditionally been limited to small-scale locus-specific or random changes aimed in a gene-centric manner, rather than enhancement or delivery of novel synthetic genetic circuits with multiple parts and control elements. Synthetic biology, on the other hand, brings engineering principles to biotechnology (*i.e.*, standardization, decoupling, abstraction and reusability) allowing a faster, more predictable,

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reproducible and ultimately cheaper ways to engineer living materials.[9] Synthetic biology has therefore been regarded as a genetic engineering in overdrive enabling, through advances in *de novo* DNA synthesis and DNA assembly,[10,11] the generation of novel proteins, regulatory elements and genetic circuits, repurposed for specific goals that are designed and engineered without being constrained by overwhelming complexity of natural cellular physiology.

Application of these principles to mammalian systems is however hampered by several obstacles. Delivery of large exogenous DNA in mammalian cells is often challenging. Furthermore, mammalian cells are transfected with expression constructs that are typically integrated randomly into the genome.[12] As a consequence, the expression of integrated transgenes will be strongly influenced by the surrounding chromatin state. Integration can cause dysregulation or accidental disruption of endogenous genes and potentially alter the behavior of the host cells.[13] Subcellular compartments, cellular differentiation, heterogeneity of individual cells, and multicellular organization result in additional layers of complexity. Predictability, delivery and prototyping of complex genetic circuits are therefore challenging.

In this review, we will underline the potential advantages of MACs used as a platform for loading and delivery of large synthetic genetic constructs into mammalian cells. MACs might provide a powerful tool for the application of synthetic biology principles to mammalian system, due to their capability of conferring the very high stability and transgene payload of a native chromosome. The generation of MACs is still a complex procedure and requires highly specialized knowledge and methodologies for design, vector manipulation and construction. However, the reduced costs in *de novo* synthesis of DNA,[11] along with the recent advances in DNA assembly technologies,[10] have already paved the way for building of entire chromosomes. The team from the J. Craig Venter Institute (JCVI) was the first to synthesize and assemble a complete bacterial genome.[14] Recently the JCVI reported the design and building a minimal bacterial genome of just 473 genes, but it contains 149 genes of unknown function.[15] This illustrates well the fact we can build a genome we do not fully understand it. Similarly, a synthetic eukaryotic chromosome has been completed and shown to be functional in a cell,[16] and work on a fully synthetic yeast genome is ongoing.[17] We need to bear in mind that this successful story is the culmination of 30 years of investigations on yeast chromosome biology since the first eukaryotic artificial chromosomes was built in budding yeast.[18,19] This effort resulted in the identification of the key determinants of chromosome generation and segregation in this lower eukaryotic system. However, crucial differences exist between bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC) and MAC formation, the most important being the strong correlation between artificial chromosome formation and epigenetic regulation, which plays a more essential role in mammalian systems.[19,20] Although plenty of insights have been already gained on factors involved in the dynamic of MAC formation, more understanding of the principal events involved would provide new opportunities to gain better control and simplify the making of new MACs.

Before describing the MACs and their potential applications, it is important to understand why scalability, orthogonality and predictability are important objectives that must be reached in order to unleash the true potential of synthetic biology in mammalian systems, and the obstacles ahead of this milestone.

Scalability. Gaining fine control over complex biological processes, such as cellular differentiation and tissue development, will likely require the design and implementation of complex genetic circuits. A necessary step in programming mammalian cell fate and responsiveness in a predictable manner is therefore the construction of complex synthetic gene networks, with a large number of regulatory elements, and effectors. In mammalian cells, more complex regulatory layers have been in part achieved by exploiting regulation of gene expression not only at transcriptional level but also at translational and post-translational level; such as employing RNA devices for small molecules or protein-responsive switch and controllable protein degradation.[21,22] Furthermore, synthetic intercellular communication systems can be designed to interconnect cells engineered with different specialized functions. Coordination of cell populations performing complementary tasks may provide a solution to overcome the limitations in single-cell rational engineering capacity, and therefore enable the design of increasingly complex multicellular gene networks.[23] However, there is still a requirement for the development of new regulatory layers that provide control on multiple gene networks. Delivering large pieces (hundreds of Kb to Mb) of exogenous DNA into the host cells and ensuring its long-term stable functionality is another major challenge associated with increased complexity.

Orthogonality, Characterization of Genetic Parts and Predictability. Applying the engineering principle of standardization of parts and assembly in the construction of genetic circuits, well-characterized genetic elements are assembled together to build simple functional modules, such as genetic switches,[24] oscillators[7] and cascades. These modules are then combined with each other to generate more complex genetic circuits. Single genetic parts or functional modules do not change their essential properties once they are part of a larger system, (ideally) independently of the cellular context, and this modularity allows reliable prediction of the behavior of a system from the interaction of its components.

Orthogonality refers to the notion that system components can be varied independently without affecting the performance of the other components and is essential for proper characterization of genetic parts and in turn for predictability of genetic circuits behavior. Having orthogonal systems that operate independently of the cell's natural machinery is also an important feature that can be valuable to reduce risks of undesirable off target effect in biomedical applications. Orthogonality is often difficult to fully attain in biological systems due to the complexity, especially in mammals. Programmable synthetic transcription factors (sTFs), new designer protein−protein interaction domains, subcellular localization of proteins hub, generation of orthogonal ribosome-mRNA pairs have been developed to generate orthogonality between different genetic circuits and the endogenous cellular processes.[25−29] The availability of well-characterized and orthogonal parts, however, is currently limiting the engineering of complex circuits in mammalian cells. Huge efforts have been made to standardize modularity in prokaryotic systems, eukaryotic systems[30] and generate orthogonality,[31,32] whereas standardization and characterization of parts on a large scale is still missing in mammalian systems, which still relies on a restricted library of orthogonal components mainly imported from microbial systems.[25]

To ensure proper characterization of genetic parts interference with the endogenous genome should be avoided when

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exogenous DNA is introduced. In mammalian cells location of genes and their chromatin state are strongly connected and mammalian genome is regulated in 3D.[33] As a consequence, the expression level of the same exogenous genes can vary depending on the integration site and the chromatin accessibility, as it is not only governed by its regulatory elements such as promoters and enhancers, but also is strongly influenced by the chromosomal context. The nature of the integration site affects transcriptional levels and stability of expression. This is called "position effect".[34] These context-dependencies drastically limit the characterization, and therefore the predictability, of parts, transcription units and synthetic circuits. Commonly used gene transfer methods, such as retroviruses, lentiviruses and transposons, are limited in delivery of specific copy numbers of the foreign DNA. The size of the payload is also often limited and repetitive sequences should be avoided—conditions difficult to fulfill for genetic circuits comprising multiple transcription units.

Recently two major developments have enhanced our ability to engineer the mammalian genome in a more efficient manner: the CRISPR/Cas system and TALENs.[35] The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Type II system or the use of Transcription Activator-Like Effector Nucleases (TALENs) enables the production of site-specific nucleases that are highly efficient. These can be used to precisely manipulate the genome, including engineering of targeted gene integration.[35] Using these technologies, stable chromosomal integration of genetic payloads into "safe harbor" sites known to tolerate the integration of transgenes (*e.g.*, AAVS1 for human and Rosa26 for mouse), can help achieve long-term stable expression.[36,37] In principle, transgenes integrated into these genomic sites are stably expressed without interfering with the activity of the endogenous genes. Orthogonality is fulfilled and deleterious consequences are avoided. However, it is arguable that no genome integration can be defined as "safe" and no genomic safe harbor has yet been fully validated.[38] Newly integrated transgenic cassettes may still influence surrounding loci and chromatin state, in undesirable ways. Although intergenic regions are often chosen for targeted transgene integration, noncoding genes are hard to detect, and low-level transcription may commonly take place in these regions.[39] Larger changes may alter chromosome trans interactions, chromosome 3D topologies,[40] interchromosomal crosstalk and the complex interplay of the genome with specific features of nuclear architecture;[41,42] such architectural control through topology-associated domains is only now being uncovered.

These may have unwanted effects, such as interactions between transcriptional activators encoded in the vector and the promoters of adjacent genes, particularly oncogenes and micro-RNA genes.[43] Reciprocally, locus specific long-range interactions can have a profound effect on expression of transgenes.[44,45] Enhancer, insulators or other long-range regulatory sequences are difficult to identify by simply looking at the genome sequence itself. Cell differentiation is a highly dynamic process with significant reconfiguration of chromosomal topologies and epigenetic states, which we are only beginning to fully appreciate; therefore, certain sites may be more appropriate integration sites in specific tissues/lineages but inappropriate in others: cellular context is the key driver.

It is because of these concerns that we would suggest that there is a clear need for an alternative delivery system for large chunks of exogenous DNA in mammalian cells. This system should not interfere with the endogenous genome, thus ensuring orthogonality. It should also provide a well-defined, stable environment for loading of exogenous DNA and, as a result, a more likely long-term stability of transgene expression. Such a system would allow proper characterization of new genetic circuits and prototyping. It should finally provide new regulatory layers for the generation of more complex genetic circuits.

EPISOMES AND MAMMALIAN ARTIFICIAL CHROMOSOME (MAC) AS ORTHOGONAL VECTORS

MACs can potentially provide an ideal platform for loading and delivery of large complex genetic circuits in mammalian cells. An artificial chromosome is complemented with components of the chromosomes that are required for segregation during mitosis and meiosis. An indispensable chromosomal element is the centromere, which is required for the attachment of chromosomes to the mitotic spindle and their segregation in mitosis. Centromere proteins (CENPs) bind to the centromeric DNA and form the scaffold for the kinetochore. The kinetochore mediates the connection between the centromere and the spindle microtubles.[20,46] Another essential component for linear chromosomes is the telomere, which consists in short repeat sequences present at the ends of the chromosomes. Special proteins bind these sequences forming a cap that prevent the chromosome ends from being recognized as double-strand breaks, avoiding chromosome fusion. The linear MAC comprises both centromere and telomeric ends. The long-term stable maintenance of the MAC is ensured by the presence of a functional centromere. The artificial chromosome is therefore stably maintained as a single copy vector without integration into the host chromosomes, allowing quantitative control on number of transgene copies.[47−49] MACs can hold large amount of DNA with theoretically no upper size limit to the DNA cloned.[50,51] Clusters of genes encoding complex pathways and complex synthetic circuits can be carried on a single MAC. As described below, MACs, once constructed, can be transferred among cell lines. Finally, lack of viral sequences minimizes adverse host immunogenic responses.

As a consequence of its properties, the MAC therefore offers an alternative orthogonal platform to accommodate large genetic circuits and minimize undesired interference with endogenous genes. Its genetic architecture is well characterized and it has been shown to be mitotically stable *in vitro* for human cells and both *in vitro* and *in vivo* in mouse cells.[52−55] It has also a system to prevent tumor formation.[47]

Episomal delivery vectors—*i.e.*, extrachromosomally maintained sequences that can be multicopy, such as Epstein−Barr virus (EBV) and scaffold/matrix attachment region (S/MAR)-based vectors—can provide an alternative to MACs to overcome the problem of position effects.[56] However, episomal vectors require components that ensure the replication and maintenance of the exogenous DNA, avoiding the loss of the vector after several rounds of cell division. This is usually done providing the vector with viral sequences that function as origin of replication.[57] These origins of replication, however, require the presence of viral proteins[58] that are potentially immunogenic. Some episomes devoid of viral elements have been developed,[59] such as S/MARs-based vectors. S/MARs mediate association with the nuclear matrix providing mitotic stability;[60] episomal vectors harboring S/MAR cosegregate with the host chromosome during mitosis.[61] However, the episomal main-

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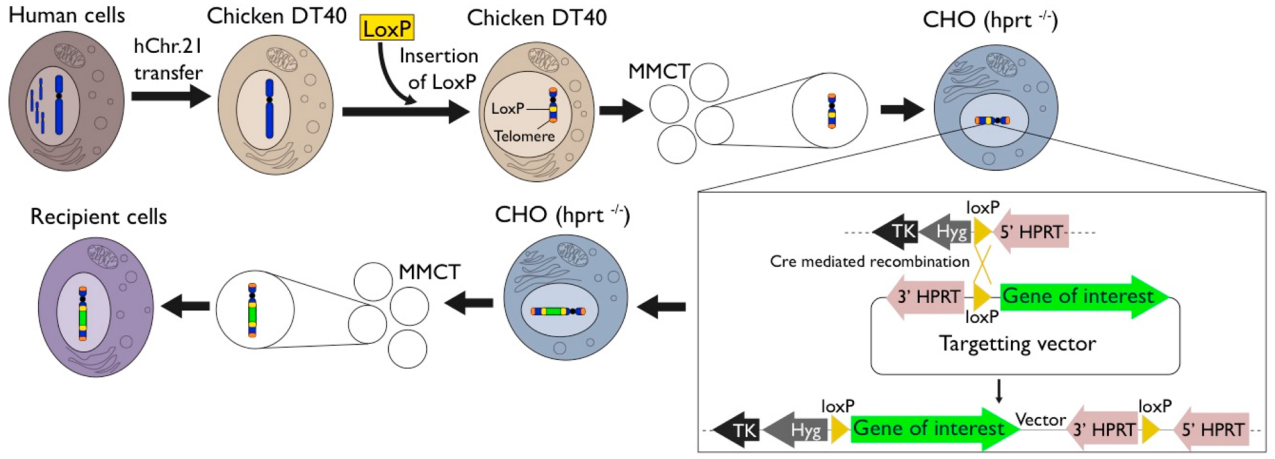

Figure 1. "Top-down" approach for generation of 21HAC. The human chromosome 21 is first transferred from human cells to recombinant-proficient chicken DT40 cells. The 21HAC is then generated by truncation of the p- and q- arms of the chromosome 21 and insertion of a loxP site into the pericentromeric region of the q-arm. The HAC chromosome is then transferred to CHO (*hprt*−/−) cells where the desired transgene can be cloned into the loxP site by Cre-mediated gene insertion with reconstitution of the HPRT gene. The HAC with the transgene is finally transferred to the desired recipient cell *via* microcell-mediated chromosome transfer (MMCT).

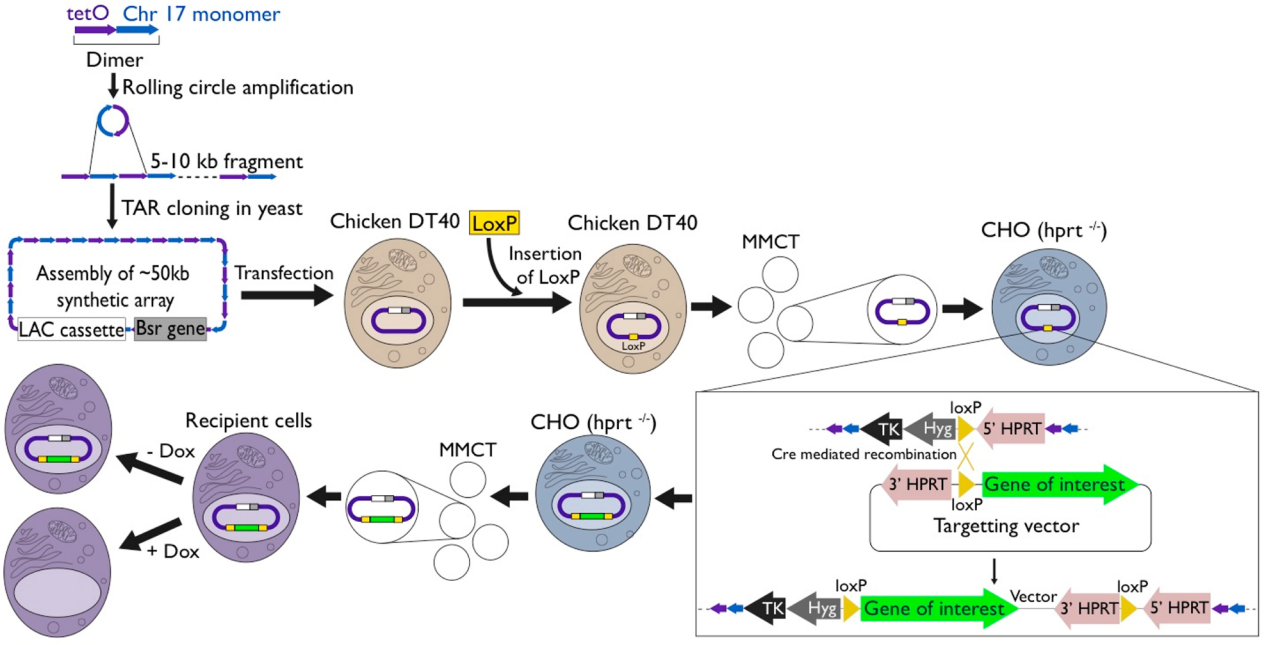

Figure 2. "Bottom-up" approach for generation of alphoid^{tetO}-HAC. Synthetic alphoid DNA array is generated by rolling circle amplification of the alphoid tetO-alphoid hChr.17 dimer up to 5−10 Kb in size. The resulting alphoid array fragment is transferred to yeast cells and introduced in a vector containing alphoid-specific hooks by TAR cloning. The resulting circular vector contains a mammalian selection marker (the Blastomicidin resistance gene) and a BAC replicon that allows a YAC clone to be transferred into *E. coli*. Transfection of mammalian cells is followed by *de novo* HAC formation. A loxP site is inserted into the alphoid^{tetO}-HAC by homologous recombination into DT40 cells. The alphoid^{tetO}-HAC is then transferred into CHO (*hprt*−/−) cells where the desired transgene can be cloned into the loxP site by Cre-mediated gene insertion with reconstitution of the HPRT gene. The HAC with the transgene is finally transferred to the desired recipient cell *via* MMCT. Following the expression of a chromatin modifier gene fused with tet-R, the HAC is maintained in the presence of doxycyclin (Dox) or the HAC is destabilized in the absence of Dox.

tenance of these vectors does not solve the problem of single copy transgenes and does not exclude the eventuality of aberrant chromosomal integration of the vector.

In the next paragraphs we discuss current strategies for MAC construction, and how MAC can function not only as an orthogonal vector, but also—due to its intrinsic characteristics—help overcome the challenges of scalability and predictability of genetic circuits in mammalian cells.

GENERATION OF MACS

MACs require a functional centromere essential to maintain their nuclear location and to ensure their correct mitotic and meiotic segregation; they can be linear or circular depending on the method used for their generation. Most of the known MACs have been built starting from human chromosome elements therefore they are named as human artificial chromosomes (HACs). Such HACs are mitotically stable in

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human cells, in mouse and in chicken cells as well; very large genomic DNA fragments have been introduce into mammalian cells using HACs.[62,63] HACs can also be transmitted through the germline—this has been shown for the murine and bovine germline[52,64−66]—providing evidence of the meiotic stability of the HACs *in vivo*. However, the retention rates of HACs were shown to be variable in mouse tissues.[67] Recently Takiguchi *et al.* reported the development of a mouse artificial chromosome employing a native mouse centromere and showed that this chromosome is stably maintained in a variety of tissues in transgenic mice with high retention rate.[55]

Two main strategies are described, focusing on the most advanced HACs generated to date (Figures 1−2). Further in depth discussions of the methodologies used to build an artificial chromosome have also been covered in several excellent recent reviews.[49,68]

Top-down Approach. The top-down approach starts with a natural human chromosome and seeks to strip it down and re-engineer large regions. The chromosome is repeatedly truncated into mini-chromosome through telomere-directed chromosome truncation in homologous recombination-proficient chicken DT40 cells.[47,69] The resulting HAC, which is a modified natural chromosome rather than a completely *de novo* synthetic chromosome, can then be transferred into other cell lines by microcell fusion. In the method known as microcell-mediated chromosome transfer (MMCT), donor cells are induced to multinucleate their chromosome and release them into microcells, which can be fused to a recipient cell line.[70] Minichromosomes have been created from human chromosomes X, Y and 14 using the top-down approach.[69,71−75] The most advanced top-down HAC is the 21HAC. It was generated starting from the human chromosome 21 through several rounds of the telomere-directed breakage removing almost all the pericentromeric regions (Figure 1).[47] The 21HAC vector was physically characterized and no known endogenous genes remained.

Bottom-up Approach. In the bottom-up approach, a *de novo* HAC is generated transfecting eukaryotic cells with synthetic alpha-satellite DNA. Human centromere DNA in made of 171 bp AT-rich alpha-satellite monomers arranged into tandem arrays that span 3−5 Mb. It is enriched with a 17-bp binding motif for the recruitment of the centromere protein B (CENP-B box), a protein involved in kinetochore assembly.[76−78] This alpha-satellite DNA has the potential for the *de novo* centromere establishment in HACs. After being transfected with 30−200 Kb alpha-DNA, by mechanisms not yet fully understood, the cell recognizes the transgenic DNA as centromeric seeding the deposition of specific protein and epigenetic markers on the exogenous DNA. These modifications will lead to the generation of a fully active centromere, thus converting the vector into an artificial chromosome.[78] The resulting HAC will be circular if the input DNA is cloned into a BAC or linear when a YAC carrying telomeric sequences is used as a vector to introduce the alphoid DNA in the target cells[79−82] (Figure 2). Alphoid DNA constructs can be efficiently delivered into target cells using a method based on herpex simplex virus type 1 (HSV-1) amplicons. This viral vector can accommodate and deliver up to 152 kb of exogenous DNA, overcoming the difficulties of introducing large alphoid DNA constructs into the cells.[83] The first "bottom-up" approach for generation of HACs was developed in human fibrosarcoma HT1080 cells.[84] All the successive methods developed for *de novo* generation of HACs were limited to the only permissive cell line HT1080.[49] This cell-type specific barrier was broken when Ohzeki and collaborators identified specific epigenetic marks associated with the alphoid DNA as responsible for the restriction of *de novo* HAC formation to HT1080 cells.[85] They demonstrated that modification of the chromatin state associated with the input alphoid DNA, is required for the formation of stable centromere, and hence HAC, in cell lines other than HT1080. *De novo* HAC generation is now possible in a wide range of cell types, including human embryonic stem (ES) cells.[86]

In the most advanced *de novo* constructed HAC, named alphoid^{tetO}-HAC, the synthetic alphoid DNA array contains tetracycline operator (tetO) sequences. The advantage of alphoid^{tetO}-HAC is that it can be easily eliminated from proliferating cells by expression of tet-repressor (tetR) fusion proteins. These proteins bind to its centromeric tetO sequences altering the chromatin and inactivating the kinetocore with subsequent loss of the HAC.[87] In a recent version, Kononenko and colleagues placed a single copy of tTA-VP64 in the HAC, avoiding the necessity of transfecting cells with potentially mutagenic retrovirus or plasmids expressing TetR. tTA-VP64 is constitutively expressed from the HAC, and after doxycycline induced activation, it generates chromatin changes in the HAC kinetochore that are not compatible with its function.[88] The alphoid^{tetO}-HAC was physically characterized. It remains stable over several months of propagation in host cells without any structural rearrangements and was not seen to integrate into the host chromosomes. The epigenetic status of the centromere remains unaltered in time, even after multiple transfers from one cell line to another.[89] The alphoid^{tetO}-HAC can be also delivered into mouse ES cells where it is stable retained throughout differentiation into somatic cell types of adult mice.[54]

Loading of Genes into HACs. Targeted integration of DNA into HAC is achieved using site-specific recombinase technology. Acceptor sites for serine or tyrosine recombinases are introduced in specific sites of the HAC by CRISPR/Cas9-mediated DNA editing. Once the HAC has been modified with the addition of acceptor sites it becomes an independent loading platform where foreign DNA can be efficiently inserted *in vivo* in single copy. The HAC, loaded with the desired transgenes, if required, can then be transferred to the target cell line. Yamaguchi *et al.* designed and generated a HAC vector containing five acceptor sites recognized by five different integrases.[90] Using this system, up to five different genes can be loaded onto the HAC. The limiting factor is that a different selection marker is needed for each DNA fragment loaded. Suzuki and colleagues developed a new method, which overcomes the problem of the scarcity in selection markers.[91] This simple method, called simultaneous or sequential integration of multiple gene-loading vectors (SIM) system, makes use of the gene trapping principle for the simultaneous or sequential integration of multiple gene-loading vectors into a HAC by reciprocal use of only two selection markers. The two selection marker genes are recycled in each sequential gene loading and deletion of selection marker genes is not required. Each recombination and incorporation of a new cassette switches the expression of selection marker genes by trapping promoter activity of the previous drug resistance gene.

RAPID AND EFFICIENT PROTOTYPING

The site-specific recombination system used to load exogenous DNA into the MAC provides a simple and efficient method for

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building of large synthetic circuits *in vivo*. The design of a new loading system, such as the SIM system discussed before, will open the possibility of sequential loading and if necessary replacement of genetic circuits allowing the progressive assembly of complex genetic circuits directly on the MAC. The foreign DNA will be inserted into the same well-characterized chromosomal context, kept as single copy DNA, resulting in generation of isogenic stable cell lines, ensuring the robust and fast prototyping of synthetic genetic networks is possible. MACs can be also employed for high-throughput screening of circuit libraries through integration of a multigene payload library.

DESIGN AND IMPLEMENTATION OF NEW REGULATORY LAYERS FOR GENE EXPRESSION

The MAC provides a platform for loading of complex genetic circuits as it can accommodate foreign DNA of large size, but it goes beyond it. The artificial chromosome as a vector offers huge potentials for the implementation of more sophisticated regulatory layers for expression of exogenous genes. We now know that the level of complexity of an organism does not relate to its number of genes. The human genome contains approximately 25 000 protein-coding genes, only about five times more than *E. coli* has, and represent less than 2% of the total human genomic sequence.[92] Pleiotropy, RNA splicing, post-translational modifications, epigenetic and chromatin remodeling provide additional parameters that contribute to increased complexity. Although gene transcription is initiated at promoters, the orchestration of transcription relies mostly on surrounding intergenic regions, *cis*-regulatory regions (*e.g.*, enhancers), and on the topological organization of the genome.[42,93,94] The eukaryotic genome is organized into functionally and structurally distinct domains as a result of intrachromosomal interactions and interactions with the nuclear lamina.[95−97] Within each domain, different epigenetic modifications prevail, resulting in a demarcation of euchromatic (transcriptionally active) and heterochromatic (transcriptionally silenced) domains. Chromatin looping brings close distal enhancer and promoters, enabling long-range transcriptional regulation.[98] By means of chromatin looping, a distal enhancer is often engaged in contacts with multiple genes, likewise, many genes form contacts with multiple distant enhancer, resulting in coregulation of multigene networks. These interactions are dynamic and change under different conditions or during cell differentiation.[42,99] Long-range transcriptional regulation by distal enhancers is therefore an important mechanism that drives the proper spatiotemporal regulation of gene expression during development.

Epigenetic modifications, chromosomal domains and long-range transcriptional regulation, are still not yet fully understood at detailed mechanistic levels; nonetheless, these might become important regulatory layers to consider when designing complex genetic circuits. The MAC provides the perfect orthogonal platform for the implementation of these regulatory events. Once generated, the chromosome can be modified within the cells. Site-specific recombination technology and CRISPR/Cas9 system would provide an efficient way to introduce user-defined sequences and to edit the DNA.[100] The artificial chromosome can therefore be regarded as an engineering chassis to build upon. Different genes or genetic circuits would be loaded at different locations within the chromosome, without interfering with the endogenous genome. The artificial chromosome DNA topology then becomes the next level of transcriptional regulation. Insulator sequences capable of recruiting the endogenous CTCF/cohesion complex would favor the formation of chromatin domain boundaries within the chromosome.[95,97] The chromatin boundaries can be then disrupted or modified targeting specific CTCF sites with inducible synthetic DNA methylases/demethylases.[101,102] The chromatin state within each domain can be dynamically modified, targeting the DNA with synthetic chromatin editors.[103] DNA-binding domains can be fused to protein domains interacting with nuclear envelope proteins and used to localize (and often silence) specific regions of the MAC in proximity of the nuclear periphery.[104] Coordinated spatial and temporal expression of multiple genes in the HAC can be achieved through chromatin looping. Inducible dimerization factors, consisting of a DNA-binding domain fused to a dimerization domain, can be employed to bring in close proximity target sequences located at long distances within the chromosome.[105] Based on this strategy, in response to a specific input, a synthetic enhancer would interact with multiple promoters at once and vice versa.

LIMITATIONS OF MACS

Despite some of the advances, the engineering of MACs is still quite difficult although several examples already exist.[68] The "top down" approach has been carried out only in recombination-proficient chicken DT40 cells, however the CRISPR-Cas9 system opens up an opportunity to attempt the breakdown of endogenous chromosome also in other types of cells and maybe to improve the process. The 21HAC derived from human chromosome 21 has been physically characterized and confirmed to be devoid of endogenous pericentromeric regions.[47] Other MACs generated using the "top down" approach should be fully characterized as residual endogenous material may result in genomic imbalances with possible consequences on the phenotype.

The "bottom up" approach relies on the quite tedious protocol of generating 30−200 Kb repetitive alpha-DNA and cloning this into a BAC/YAC vector; the vector is then isolated and delivered into the target cell. However, processing of BAC/YAC vectors is very inefficient, as large circular DNA molecules tend to randomly break and linearize during DNA transfection. Using other method of delivery such as fusion of yeast spheroblasts containing the YAC vector with mammalian target cells may simplify the process.[106] The HAC formation is always accompanied by multimerization of the input alphoid DNA.[89] Multimerization of the input DNA may therefore represent an obligate step in MAC formation. These rearrangement events result in structurally uncharacterized artificial chromosomes and raise the issue on how to get better control on MAC formation to avoid this problem. The process that leads to the generation of a new centromere is not completely understood. In rare instances, when a human chromosome loses its centromere due to chromosome breakage, new functional centromeres, called neocentromeres, can be generated on a nonalpha satellite DNA.[107] These events suggest that there is no absolute requirement for any particular DNA sequence for the generation of a centromere. Centromeric loci are rather defined by specific epigenetic marks that are not specified by the primary DNA sequence.[19,20] MAC formation is therefore strongly correlated with epigenetic regulation. Better understanding of the elusive epigenetic state of the centromere may provide new means to turn normal chromatin into centrochromatin through a more controllable process.

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Safe, streamlined and efficient procedures for MAC transfer are still an important outstanding challenge for the future. Although recent progress in the field have made possible the targeting of different cell types, included fibroblasts,[108] and therefore possible the targeting of desired cells *in vivo*, the method is still tedious and restricted to a few numbers of donor cells suitable for microcell formation. Cloning and moving the HACs *via* MMCT takes months and requires an expertise rarely found in a standard laboratory. All these challenges call for interdisciplinary teams to work together to address, and together to push this field forward.

CONCLUSIONS

Our ability to effectively manipulate mammalian cells with fully synthetic DNA elements at large scale would have myriad possibilities for biotechnological and biomedical applications.[109] The next-generation genetic circuits have the potential to provide more precise and sophisticated control on gene expression coupling sensing and delivery mechanisms and opening up new exciting possibilities in generation of synthetic multicellular networks. Circuits have already been designed for diverse purposes, such as treating of metabolic disorder using biosensor cells,[110] or killing of cancer cells through cell death promoting genetic sensor designed to detect specific sets of cancer-associated signals[111,112] or through chimeric antigen receptor (CAR) T-cell therapies.[113] The development of synthetic biology in mammalian systems would also enable a more systematic investigation on epigenetic mechanisms and spatial regulation of gene expression. This in turn translates in the possibility of higher control on induced pluripotent stem cell (iPSC) differentiation to produce and engineer autologous cells from patients[114] for application in basic biology, disease modeling, drug screening[115] and new developments in regenerative medicine. There is therefore great potentiality in mammalian systems for engineering of new functions and genetic devices, but also for gaining new insights into the mechanisms operating in native cell systems.

In this promising avenue the MACs represent a new class of promising vector for multiple applications. They bypass many problems associated with existing viral and nonviral systems. MACs have indefinite cloning capacities, copy number control, long-term gene expression, and they are mitotically and meiotically stable in the absence of selection. Potentially any combination of genes can be cloned into the MAC and transferred into a recipient target cell type. MACs have already been used for gene function studies and are suitable vector for gene therapy although further investigation is required to evaluate efficiency and safety of MAC delivery, to cell cultured *in vitro* but also *in vivo* using animal models.

MACs are also a potential platform for engineering of a more sophisticated gene expression control in mammalian cells. In combination with programmable synthetic transcription factors, improved DNA synthesis and assembly, plus genome editing technologies, MACs offer great opportunities for mammalian synthetic biology. Complex genetic circuits can be designed and built that impart spatial and temporal control over the endogenous cellular genetic program. This strategy could eventually prove to be a more efficient way to directly convert fibroblasts into different cell types and to manipulate basic cell behaviors such as proliferation, cell death, fusion, polarization, locomotion, cell−cell adhesion, cell−cell communication, that would allow 'programmable morphogenesis' for tissue generation. Exogenous biosynthetic pathways can be assembled into the HAC to produce diverse compounds or complex protein therapeutics. The HAC can be used to introduce genetic constructs and pathways to test their effect on a certain disease or drug treatment.

In summary, generation of MACs using simple methods of manipulation and transfection holds great potential for synthetic biology designs in mammalian cell lines and future applications such as cell line generation, generation of transgenic animals, human gene therapy and regenerative medicine.

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Notes

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ABBREVIATIONS

MAC:Mammalian Artificial Chromosome
HAC:Human Artificial Chromosome
CAR:chimeric antigen receptor
MMCT:microcell-mediated chromosome transfer
SIM:simultaneous or sequential integration of multiple gene-loading vectors system
CTCF:CTCC-binding factor
CRISPR:Clustered Regularly Interspaced Short Palindromic Repeats Type II system
TALEN:Transcription Activator-Like Effector Nucleases
CENP:centromere proteins
S/MARs:scaffold/matrix attachment regions

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