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Promising Role of Engineered Gene Circuits in Gene Therapy

Wei-dong Wang and Jinyi Lang
Department of Radiation Oncology, Sichuan Cancer Hospital
P.R. China

1. Introduction

Synthetic biology is concerned with applying the engineering paradigm of systems design to biological systems in order to produce predictable and robust systems with novel functionalities that do not exist in nature. The circuit-like connectivity of biological parts and their ability to collectively process logical operations was first appreciated nearly 50 years ago. This inspired attempts to describe biological regulation schemes with mathematical models and to apply circuit analogies from established frameworks in electrical engineering(McAdams & Arkin A,2000). Meanwhile, breakthroughs in genomic research and genetic engineering (e.g., recombinant DNA technology) were supplying the inventory and methods necessary to physically construct and assemble biomolecular parts. As a result, synthetic biology was born with the broad goal of engineering or "wiring" biological circuitry—be it genetic, protein, viral, pathway, or genomic—for manifesting logical forms of cellular control. Synthetic biology, equipped with the engineering-driven approaches of modularization, rationalization, and modeling, has progressed rapidly and generated an ever-increasing suite of genetic devices and biological modules.

synthetic biology is seeking to use and expand the mechanisms that control biological organisms using engineering approaches. These approaches will be applied on all scales of biological complexity: from the basic units to novel interactions between these units to novel multi-component modules that generate complex logical behaviour, and even to completely or partially engineered cells(McAdams & Shapiro,1995). Bringing the engineering paradigm to biology will allow us to apply existing biological knowledge to biotechnological problems in a much more rational and systematic way than has previously been possible, and at the same time to expand the scope of what can be achieved this way. The introduction of design principles such as modularity of parts, standardization of parts and devices according to internationally recognized criteria, and the adaptation of available abstract design procedures to biological systems, coupled to novel technological breakthroughs that allow the decoupling of design and fabrication, will fundamentally change our current concepts of how to manipulate biological systems. In this sense, synthetic biology is not primarily a "discovery science", but is ultimately about a new way of making things. By adapting natural biological mechanisms to the requirements of an engineering approach, the possibilities for re-assembling biological systems in a designed way will increase tremendously. While several of the fundamental scientific issues and current applied objectives of synthetic biology overlap with those in other, more mature fields, especially

biotechnology and systems biology, synthetic biology should be properly seen as a completely new discipline, which brings a systematic, application-driven engineering perspective to biology. Just as in chemistry about a century ago, biology now seems poised to enter an era where significant advances in understanding will derive from a fruitful dialogue between theory and experiment, from analytical and synthetic efforts, and from interdisciplinary interaction with the chemical, physical, engineering and computational sciences. The potential for interaction with nanotechnology is especially apparent and appealing. It is often said that biology is the only existing nanotechnology that really works. But if we want to exploit this 'natural nanotechnology' for applied, engineering objectives, we will ultimately need to be able to intervene and to modify it at the level that synthetic biology is exploring. It can be anticipated that the major change that the field of synthetic biology will bring is the synergistic integration of existing disciplines: not just biology and engineering, but also computer modelling, information technology, control theory, chemistry and nanotechnology. Ulitmately, it is likely that the analytical and synthetic approaches to biology, as well as the in vitro and in vivo approaches, will fully complement each other.

Synthetic biology will revolutionize how we conceptualize and approach the engineering of biological systems. The vision and applications of this emerging field will influence many other scientific and engineering disciplines, as well as affect the next generation of cancer therapy. In this article, we discuss and analyze the recent advances in synthetic biology towards engineering complex living systems through novel assemblies of biological molecules. The discovery of mathematical logic in gene regulation in the 1960s (e.g. the lac operon; Monod and Jacob, 1961) and early achievements in genetic engineering that took place in the 1970s, such as recombinant DNA technology, paved the way for today's synthetic biology. Synthetic biology extends the spirit of genetic engineering to focus on whole systems of genes and gene products. The focus on systems as opposed to individual genes or pathways is shared by the contemporaneous discipline of systems biology, which analyzes biological organisms in their entirety. Synthetic biologists design and construct complex artificial biological systems using many insights discovered by systems biologists and share their holistic perspective. It is useful to apply many existing standards for engineering from well-established fields, including software and electrical engineering, mechanical engineering, and civil engineering, to synthetic biology. Methods and criteria such as standardization, abstraction, modularity, predictability, reliability, and uniformity greatly increase the speed and tractability of design. However, care must be taken in directly adopting accepted methods and criteria to the engineering of biology. We must keep in mind what makes synthetic biology different from all previous engineering disciplines. The insight gained from fully appreciating these differences is critical for developing appropriate standards and methods. Building biological systems entails a unique set of design problems and solutions. Biological devices and modules are not independent objects, and are not built in the absence of a biological milieu. Biological devices and modules typically function within a cellular environment. When synthetic biologists engineer devices or modules, they do so using the resources and machinery of host cells, but in the process also modify the cells themselves. A major concern in this process is our present inability to fully predict the functions of even simple devices in engineered cells and construct systems that perform complex tasks with precision and reliability. The lack of predictive power stems from several sources of uncertainty, some of which signify the incompleteness of available information about inherent cellular characteristics. The effects of gene expression

noise, mutation, cell death, undefined and changing extracellular environments, and interactions with cellular context currently hinder us from engineering single cells with the confidence that we can engineer computers to do specific tasks. However, most applications or tasks we set to our synthetic biological systems are generally completed by a population of cells, not any single cell. In a synthetic system, predictability and reliability may be achieved in two ways: statistically by utilizing large numbers of independent cells or by synchronizing individual cells through intercellular communication to make each cell more predictable and reliable. More importantly, intercellular communication can coordinate tasks across heterogeneous cell populations to elicit highly sophisticated behavior(Khalil & Collins, 2010). Thus, it may be best to focus on multicellular systems to achieve overall reliability in performing complex tasks.

2. Recent advancements in synthetic biology

2.1 Engineering of artificial gene networks

Significant efforts were recently undertaken in the design of artificial genetic networks in prokaryotic and eukaryotic systems. Here, different genetic elements or 'parts' are (ultimately) rationally combined to 'devices' to realize specific cellular behaviors that have frequently analogies to elements from electric circuits such as switches and oscillators. We will outline recent efforts in the development of artificial gene networks.

2.1.1 Switches

A switch lets the cell adopt one of two possible states, depending either on the presence or absence of a chemical inducer or on two separate external stimuli (toggle switch) (Becskei et al.,2001). The latter behavior can be easily designed from any two repressors that reciprocally inhibit the transcription of their genes (Gardner et al.,2000). Switching between states can be achieved by intermittently inactivating the repressor that maintains the current state (such as adding a chemical inducer or increasing the temperature). Essentially, this property conveys a cell with a memory of its previous cultivation history and thus represents an epigenetic toggle switch. The former behavior requires positive feedback in the regulatory processes, such as (1) the positive autoregulation of a positive regulator's gene transcription or (2) the concomitant upregulation of an operon by external inducer and of the gene that encodes the transporter protein for entrance of the inducer. Besides the artificial design of such systems, this behavior is rather common in a number of well-characterized bacterial expression systems such as the bacterial lactose and arabinose systems (Atkinsonet al., 2003;Ozbudak et al.,2004;Vilar et al.,2003).

In addition, the switches can be engineered with a hysteretic character, so that the system switches into the 'ON' state at a higher concentration of external signal than is required to switch back to the 'OFF' state. This requires that the concentration of activator or active repressor can be made a function of the history of the cell, e.g. by adding another regulatory layer on top of the positive feedback element. This can be a concentration-dependent inactivation of a repressor that competes with an activator. Depending on the previous state of the cell, a given concentration of active repressor interacts with either high or low concentrations of activators, leading to a differentiation in response depending on the history (Kramer & Fussenegger, 2005).

2.1.2 Complex networks

An oscillator produces regular fluctuations in network elements such as reporter proteins. Oscillators have been realized in two ways: as ring oscillators ('repressilators') or as a combination of activation and repression elements. The ring oscillator consists of three repressor genes that are coupled to three corresponding promoters in such a way, that each repressor protein can turn off the synthesis of one other repressor protein. This design worked on single cell level, but not on culture-level, which probably has to do with the noise involved on gene expression level (Elowitz & Leibler,2000). However, by combining positive and negative regulation, it is possible to reduce the noise to such a degree that population-synchronized oscillation behavior over three periods can be observed in a turbidostat. Interestingly, such oscillating systems can be extended to include metabolite concentrations (Fung et al.,2005).

In order to execute ever more complex logical behavior, it will be important to be able to 'integrate' more and more signals into determining one or more cellular functions. This is facilitated by the high level of modularity in the regulatory elements of eukaryotic systems. This modularity makes them particularly amenable to design and can be used to implement a wide variety of logical behaviors for two and three signal inputs while exploiting only a limited number of genetic elements (Kramer et al.,2004).

2.1.3 Networks for intercellular communications

Creating macroscopically observable artificial functional behavior in a cell population requires some kind of synchronization. Such synchronization can be enforced by adding chemical inducers or by letting the cells themselves produce a signal in response to a change in a culture property. One example for such a property is cell density which can be communicated by quorum sensing, for example via the *luxR/luxI* system of *Vibrio fischeri* or via artificially engineered systems (Bulter et al., 2004).

The *luxR*/*luxI* system has been used to trigger a variety of population–density dependent responses, such as flipping of a toggle switch (Kobayashi et al., 2004) or programmed population control (You et al., 2004). The system has also been exploited to design spatial patterns of behavior that re-build aspects of multicellular systems: when producer cells send the autoinducer signal of the *lux* system via diffusion through a plate, cells at different distances from the senders experience differently steep gradients once the autoinducer reaches them. Alternatively, cells can be used to detect the differences in inducer concentration in resulting (quasi-)steady state. Networks can be designed which are able to detect these rather subtle differences in environmental conditions and which translate them into adequate cellular responses such as different pulses of reporter proteins or stable colorimetric patterns (Basu et al.,2004;Basu et al., 2005), introducing space as an additional design parameter into the synthetic biology realm.

2.1.4 Issues related to the design of genetic circuits

For the design of genetic networks, the availability of functional elements with specific properties (such as binding constants and degradation rates) that fit the design purpose is crucial. So far, we are only at the beginning of being able to easily measure, let alone program kinetic parameters, co-operativities or binding constants. Consequently, the design process remains–for the time being–an iterative process that still contains considerable elements of trial and error. Nevertheless, some work-around tools are available today in

order to, at least crudely, shift certain characteristics from wild-type values to values that allow a desired behavior to be implemented. These include variations in gene dosage via changes in plasmid replicon, the increase of protein degradation rates by fusion to suitable protease sensitive tag-sequences, variations in the strength of RBSs (Yokobayashi et al.,2002) or drawing on the large number of mutants that are available for a number of model systems (such as phage λ, the *lac* system or the *tet* system). Alternatively, parameters can be adapted to the desired behavior by directed evolution, if a suitable assay is available. However, it is not really clear how such directed evolution assays can be easily tailored to screen for relatively subtle differences in properties important for optimized design. In summary, a primary task for the immediate future is to gain access to complete system parameter sets, which can then serve as the starting point to produce parts with parameter values that span suitable ranges.

2.2 Engineering of systems

Synthetic biology is a very young discipline that follows a powerful technological vision. However, there are no examples available where the whole approach has been implemented. Still, in some cases specific aspects of synthetic biology have been of critical importance. We will discuss the following examples: the design of an *E.coli* capable of image processing, refactoring of the phage T7, the design of novel polyketide antibiotics and the manufacturing of precursors for the anti-malaria drug artemisinin.

An original example for new applications that derives from the interface of engineering and life sciences, which came out of the iGEM student competition, is the image-processing *E.coli*. By designing proteins that couple light-detection to well-known *E.coli* regulatory circuits, first steps towards light-detecting pixel sizes of micrometer dimensions are possible (Levskaya et al., 2005).

A more fundamental aspect is covered by the work on the phage T7, which tries to help to answer the question whether it is indeed possible to refactor significant portions of small genomes. In other words, can we indeed modify those genomes according to the requirements of 'engineerability' such as monofunctionality of a part of the sequence and organization of the DNA into functional segments. Refactoring 10 kb of the T7 genome, representing about a quarter of the total genome, still produces functional phages, though their efficiency in propagation is reduced (Chan et al., 2005). This is an important validation of the synthetic biology approach, even though on a small scale. It remains to be seen whether the same concepts can be applied to more complex systems such as microbes.

Two examples for application of synthetic biology concepts come from the area of pharmaceutical production and involve primarily the opportunities offered by *de novo* DNA synthesis, such as the direct adaptation of codon usage, implementation of suitable regulatory circuitry and the possibility to modularize the DNA sequences by restriction sites to facilitate iterative optimizations. The first example involves the adaptation of polyketide synthesis to well studied *E.coli* production strains and the subsequent design of novel polyketides by semi-randomized recombination of polyketide synthase genes. These recombinations were easily enforced along the interfaces of the different functional modules that make up a synthase and resulted in a rather high success rate of detecting novel polyketides (Menzella et al., 2005).

Along similar lines, another project that very much catches the spirit of synthetic biology is the construction, from scratch, of a cheap terpenoid production pathway in *E.coli* leading to

artemisinic acid, a precursor to the anti-malaria drug artemisinin. This goal essentially requires the design of an entirely new pathway in a suitable production organism. The corresponding pathway elements can be recruited from bacteria (*E.coli*), yeast (*Saccharomyces cerevisiae*) and plant (*Artemisa annua*), redesigned and functionally expressed in bacteria or yeast, effectively paving the road to a low-cost production route to effective malaria treatment (Martin et al.,2003;Ro et al.,2006).

Although the design of novel biological systems is only beginning, all ingredients of the engineering approach are visible: the role of *de novo* DNA synthesis, the design of well-behaved parts on the DNA and protein level, the organization of parts into the next functional level of devices and the corresponding abstractions and the attempt to introduce standardization, even though for the time being only on a parts level. With the design of ever more complex systems, the need to emphasize these elements will undoubtedly increase.

3. Design strategies of synthetic genetic circuits

Synthetic biology encompasses the building of novel biological entities for useful purposes and the corresponding endeavors can be subdivided into two distinct types of tasks: systems design and systems fabrication. Here, we will discuss the essential elements of these two tasks with a special focus on the computational and informatics requirements.

Fabrication deals with the transformation of design plans into actual physical instances. Today, this still involves a significant amount of cloning work, which should decrease in the future due to *de novo* DNA synthesis. The fabrication as such, is not expected to create a great demand for novel informatics tools.

In contrast, systems design consisting of forward-engineering of biological parts, devices or systems strongly relies on computing and informatics tools that assist the design process. Ultimately, it would be desirable to have computer aided design tools—CAD tools for biological engineering—in analogy to the respective software tools in the areas of mechanical or civil engineering. Using such software, the synthetic biology design engineer would try to improve the behavior of a biological system *in silico* by optimizing design parameters targeting a selected objective function. Design variants would be tested computationally by means of simulations.

Such design tools will be based on quantitative mechanistic models that reproduce biological behavior and–in order to be useful for forward-engineering design—would also have predictive power. In biology, we have not yet reached a level of understanding where such models can be developed on a large scale and consequently, true biological engineering is hardly possible until now (Endy & Brent, 2001). In fact, in most cases today, we are faced with highly uncertain or even unknown model topologies, mechanisms and parameters. The recent advances in the post-genomic research and especially in systems biology, however, provide hope that sooner or later we will be able to draw on a body of knowledge that allows for the envisioned directed engineering of biology (Endy & Brent, 2001). Ultimately, mathematical models developed for research purposes (e.g. in systems biology) will be employed as design models in synthetic biology. In contrast to the current lack of predictive models, tools for modeling and simulation exist in large numbers (Lemerle et al, 2005).

We envision that in the long run we will require models and design software for the following tasks: (1) sequence–based (*ab initio*) prediction of structure, function and interactions of macromolecules, in particular proteins and mRNA, (2) prediction of the

dynamics of signaling and regulatory networks; and (3) prediction of the dynamics of metabolic networks. For each of these areas, we will shortly sketch the current status of development and also elaborate on future tasks.

3.1 Design of functions and interactions of macromolecules

We would like to predict—starting from a linear sequence of nucleotides or amino acids—2D (mRNA) and 3D structures of the respective macromolecules (RNA, proteins), as well as their function and their interaction parameters with other cellular components (DNA, metabolites, etc.). In other words, as outlined above we would like to have the possibility to modify sequences in a targeted manner to obtain, e.g. novel transcription factors (i.e. with altered binding constants or kinetics) or proteins with novel functions.

However today, as an example *de novo* protein structure prediction from a linear amino acids sequence can only be achieved for small protein domains at significant computational costs (Bradley et al.,2005; Misura et al.,2006). Nevertheless, starting from known structures of 'scaffold' proteins, design methods are available, which can be used to rationally modify the proteins' structure and function, i.e. to build completely new active sites into proteins or to redesign binding specificities of proteins. However, such design processes still go through several cycles of iterative improvement involving design, analysis, redesign, etc. where computational tools such as FoldX (Schymkowitz et al.,2005) are typically employed. In other words, the design of tailored catalytic activities on artificial proteins seems to be within reach, while quantitative prediction of enzymatic activity and selectivity from 3D protein structures in general is not yet feasible. For further information on the current status in modeling of protein structures and interactions, the reader is referred to a recent review (Schueler-Furman et al.,2005).

Based on structure models, molecular dynamics simulation have shown to be a versatile tool to investigate the dynamic behavior of complexes between DNA binding sites and respective DNA target sites (Marco et al., 2003;;Obika et al.,2003). These tools can also be employed to predict the effect of structural modulations on protein–ligand interactions in a way that would allow forward-engineering design of, e.g. DNA-binding specificity of transcription factors.

3.2 Design of signaling and regulatory networks

Artificial signaling and regulatory gene networks will need to be assembled for synthetic biology. Today, such circuits are still frequently assembled by intuition and optimized through several rounds of trial and error (Kærn et al.,2003) and the mathematical models are only developed once proper *in vivo* function has been demonstrated. Deterministic or stochastic models (or a combination of both) are then used to describe the observed dynamic behavior of the circuit.

Ideal, however, would be models that allow deriving *in silico* suggestions for optimal design strategies or debugging, prior to implementation of the circuit *in vivo* (Sprinzak & Elowitz, 2005). Such models should be able to capture the dynamic behavior of the gene networks. In cases where only small molecule numbers are involved (as in gene transcription or translation, where transcription factors and mRNA molecules only occur in low copy numbers), the models would also need to be able to reproduce the inherent stochasticity of such processes. This is imperative as it was shown that stochasticity in combination with certain system architectures can result in different system states (Pedraza & van

Oudenaarden,2005). A robust design of new devices and systems must exclude such eventualities.

In summary, to enable the envisioned forward-engineering (model-based) design of signaling and regulatory circuits, improvements are required in the following areas: It is necessary (1) to obtain an improved quantitative understanding of regulatory and signaling processes; (2) to develop effective rules (Wall et al.,2003) and standards for characterizing modules and (3) to improve multiscale simulation algorithms as the existing ones are limited in a way that the participating reactions have to occur on a comparative time scale and the participating reaction species have to fulfill certain population size requirements.

4. Genetic circuits and therapeutics

4.1 Drug target identification

Building up synthetic pathways and systems from individual parts is one way of identifying disease mechanisms and therapeutic targets. Another is to deploy synthetic biology devices to systematically probe the function of individual components of a natural pathway. To achieve post-transcriptional control over a target gene, the mRNA sequence of its 5′-UTR was designed to form a hairpin structure that sequesters the ribosomal binding site (RBS) and prevents ribosome access to it. Translational repression of this *cis*-repressed mRNA could then be alleviated by an independently regulated *trans*-activating RNA that targets the stem-loop for unfolding. Engineered riboregulators were used in a subsequent study to tightly regulate the expression of CcdB, a toxic bacterial protein that inhibits DNA gyrase, so as to gain a better understanding of the sequence of events leading to induced bacterial cell death(Dwyer,2007). These synthetic biology studies, in conjunction with systems biology studies of quinolones (antibiotics that inhibit gyrase), led to the discovery that all major classes of bactericidal antibiotics induce a common oxidative damage cellular death pathway(Kohanski,2008). This work provided new insights into how bacteria respond to lethal stimuli, and paved the way for the development of more effective antibacterial therapies.

Once a faulty pathway component or target is identified, whole-cell screening assays can be designed using synthetic biology strategies for drug discovery. As a demonstration of this approach, Fussenegger and colleagues(Weber,2008)developed a synthetic platform for screening small molecules that could potentiate a *Mycobacterium tuberculosis* antibiotic. Ethionamide, currently the last line of defense in the treatment of multidrug-resistant tuberculosis, depends on activation by the *M. tuberculosis* enzyme EthA for efficacy. Due to transcriptional repression of *ethA* by the protein EthR, however, ethionamide-based therapy is often rendered ineffective. To address this problem, the researchers designed a synthetic mammalian gene circuit, featuring an EthR-based transactivator of a reporter gene, and used it to screen for and identify EthR inhibitors that could abrogate resistance to ethionamide. Importantly, because the system is a cell-based assay, it intrinsically enriches for inhibitors that are nontoxic and membrane-permeable to mammalian cells, which are key drug criteria as *M. tuberculosis* is an intracellular pathogen. This framework, in which drug discovery is applied to whole cells that have been engineered with circuits that highlight a pathogenic mechanism, could be extended to other diseases and phenotypes.

4.2 Therapeutic treatment

Synthetic biology devices have additionally been developed to serve as therapies themselves. Entire engineered viruses and organisms can be programmed to target specific

pathogenic agents and pathological mechanisms. In two separate studies(Lu,2009), for instance, engineered bacteriophages were deployed to combat antibiotic-resistant bacteria, by endowing them with genetic mechanisms that target and thwart bacteria's antibiotic evasion techniques. The first study was prompted by the observation that biofilms, in which bacteria are encapsulated in an extracellular matrix, have inherent resistance to antimicrobial therapies and are implicated sources of persistent infections. To more effectively penetrate this protective environment, T7 phage was engineered to express the biofilm matrix-degrading enzyme dispersin B (DspB) upon infection (Lu,2007). The two-pronged attack of phage-induced lysis fueling the creation and spread of matrix-degrading enzyme resulted in 99.997% removal of biofilm bacterial cells.

It was hypothesized that inhibition of certain bacterial genetic programs could help current antibiotic therapies achieve more effective activity. In this case, bacteriophages were deliberately designed to be non-lethal so as not to elicit resistance mechanisms; instead, non-lytic M13 phage was used to suppress the bacterial SOS DNA damage response by overexpression of its repressor, *lexA3*. The engineered bacteriophage significantly enhanced killing by three major classes of antibiotics in traditional cell culture and in *E. coli*-infected mice, potentiated killing of antibiotic-resistant bacteria, and importantly reduced the incidence of antibiotic-induced resistant cells.

Synthetically engineered viruses and organisms that are able to sense and link their therapeutic activity to pathological cues may be useful in the treatment of cancer, where current therapies often indiscriminately attack tumors and normal tissues. Adenoviruses, for instance, were programmed to couple their replication to the state of the p53 pathway in human cells(Ramachandra,2001). Normal p53 production would result in inhibition of a critical viral replication component, whereas a defunct p53 pathway, which is characteristic of tumor cells, would allow viral replication and cell killing. In another demonstration of translational synthetic biology applied to cancer therapy, Voigt and colleagues()developed cancer-targeting bacteria and linked their ability to invade the cancer cells to specific environmental signals. Constitutive expression of the heterologous *inv* gene (from *Yersinia pseudotuberculosis*) can induce *E. coli* cells to invade both normal and cancer human cell lines. So, to preferentially invade cancer cells, the researchers placed *inv* under the control of transcriptional operons that are activated by environmental signals specific to the tumor microenvironment. These engineered bacteria could be made to carry or synthesize cancer therapies for the treatment of tumors.

In addition to engineered therapeutic organisms, synthetic circuits and pathways can be used for the controlled delivery of drugs as well as for gene and metabolic therapy. In some cases, sophisticated, kinetic control over drug release in the body may yield therapeutic advantages and reduce undesired side effects. Most hormones in the body are released in time-dependent pulses. Glucocorticoid secretion, for instance, has a circadian and ultradian pattern of release, with important transcriptional consequences for glucocorticoid-responsive cells(Anderson,2006). Faithfully mimicking these patterns in the administration of synthetic hormones to patients with glucocorticoid-responsive diseases, such as rheumatoid arthritis, may decrease known side effects and improve therapeutic response. Periodic synthesis and release of biologic drugs can be autonomously achieved with synthetic oscillator circuits or programmed time-delay circuits(Weber,2007) In other cases, one may wish to place a limit on the amount of drug released by programming the synthetic system to self-destruct after a defined number of cell cycles or drug release pulses.

Gene therapy is beginning to make some promising advances in clinical areas where traditional drug therapy is ineffective, such as in the treatment of many hereditary and metabolic diseases. Synthetic circuits offer a more controlled approach to gene therapy, such

as the ability to dynamically silence, activate, and tune the expression of desired genes. In one such example, a genetic switch was developed in mammalian cells that couples transcriptional repressor proteins and an RNA interference (RNAi) module for tight, tunable, and reversible control over the expression of desired genes. This system would be particularly useful in gene silencing applications, as it was shown to yield > 99% repression of a target gene. Additionally, the construction of non-native pathways offers a unique and versatile approach to gene therapy, such as for the treatment of metabolic disorders. Operating at the interface of synthetic biology and metabolic engineering, for instance, Liao and colleagues(Dean,2009) recently introduced the glyoxylate shunt pathway into mammalian liver cells and mice to explore its effects on fatty acid metabolism and, more broadly, whole-body metabolism. Remarkably, the researchers found that when transplanted into mammals the shunt actually increased fatty acid oxidation, evidently by creating an alternative cycle. Furthermore, mice expressing the shunt showed resistance to diet-induced obesity when placed on a high-fat diet, with corresponding decreases in total fat mass, plasma triglycerides, and cholesterol levels. This work offers a new synthetic biology model for studying metabolic networks and disorders, and for developing treatments for the increasing problem of obesity.

5. Challenges and future directions

Constructing a functional synthetic circuit requires assembling diverse genetic elements and getting them to work together. In general, combining disparate components requires the tuning of biochemical parameters such as affinities or rate constants, which is often difficult to implement in biological circuits. Characterization of a component may be valid in one context (locus, plasmid, strain, environment, and so on), but not in others. How can one design an operating circuit given these limitations? Several strategies have been applied. First, the use of tunable elements, such as transcription factors derived from tetR(Lutz,1997), allows external control over some parameters. Second, one can screen libraries of mutated components, or apply directed evolution in the laboratory, to optimize parameters. A third strategy is to use robust circuit designs that are inherently insensitive to unknown or variable parameters. Such designs are particularly interesting because they may have been selected by evolution for the very same property(Barkai,1997).

A related challenge is computational modelling of genetic circuits. Modelling is essential both for analysis of natural systems and also for design of synthetic ones. However, several problems complicate its application to cellular circuits. These include parameter sensitivity, the lack of effective rules to simplify complex circuits, and the difficulty of incorporating extrinsic noise. Because synthetic circuits are simpler and better characterized than their natural counterparts, they will probably offer ideal test systems to develop and refine models. The results should apply both to natural and synthetic circuits.

What are the goals of the synthetic circuit paradigm outlined here? One is to better understand natural circuits by building minimal replicas of those circuits, observing their dynamics in vivo, and comparing them to one another and to their natural counterparts.

The synthetic circuits presented above are highly simplified. However, as we gain confidence and expertise in our ability to build, model and analyse these circuits, we will be able to construct replicas of greater verisimilitude. Possible natural circuits that could be investigated this way include decision making in response to stress and DNA damage, as in the natural p53/mdm2 circuit(Vogelstein,2000), differentiation in response to extracellular signals, as in oocyte maturation(Xiong,2003), and regulated temporal oscillations, as in the

cell cycle and circadian clock. Circuits that use the intrinsically noisy nature of the cell to create probabilistic behaviours are particularly compelling examples.

A second goal is to discover what other, non-natural, circuit designs are possible given realistic biological components, and which of those operate reliably in vivo. This will be achieved by building and characterizing a variety of alternative circuit designs in living cells. In this way, one may ask what advantages naturally evolved circuits have over synthetic ones. For example, the synthetic clock designs described earlier have not been discovered to occur in nature, suggesting that natural designs may confer better performance. At the same time, non-natural designs may prove useful for biotechnology applications.

Perhaps the most intriguing problem is how a circuit operates in the context of a complete organism. There are no dotted lines inside the cell isolating circuits from one another. The ultimate test for this synthetic approach is to delete natural circuits and replace them with synthetic counterparts within organisms. This will require synthetic circuits to interface with the rest of the cell. For example, by replacing the Drosophila circadian clock with synthetic versions we could learn more about the interaction of the circadian module with other functional subsystems in the organism. Even the most optimistic synthetic biologist would expect such circuits to be less functional than their natural counterparts. However, perhaps at this stage one can learn more by putting together a simple, if inaccurate, pendulum clock than one can by disassembling the finest Swiss timepiece.

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The aim of this book is to cover key aspects of existing problems in the field of development and future perspectives in gene therapy. Contributions consist of basic and translational research, as well as clinical experiences, and they outline functional mechanisms, predictive approaches, patient-related studies and upcoming challenges in this stimulating but also controversial field of gene therapy research. This source will make our doctors become comfortable with the common problems of gene therapy and inspire others to delve a bit more deeply into a topic of interest.

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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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