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Review

Gene-circuit therapy on the horizon: Synthetic biology tools for engineered therapeutics*

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Therapeutic genome modification requires precise control over the introduced therapeutic functions. Current approaches of gene and cell therapy fail to deliver such command and rely on semi-quantitative methods with limited influence on timing, contextuality and levels of transgene expression, and hence on therapeutic function. Synthetic biology offers new opportunities for quantitative functionality in designing therapeutic systems and their components. Here, we discuss synthetic biology tools in their therapeutic context, with examples of proof-of-principle and clinical applications of engineered synthetic biomolecules and higher-order functional systems, i.e. gene circuits. We also present the prospects of future development towards advanced gene-circuit therapy.

Keywords: synthetic biology, gene circuit, gene therapy, cell therapy, gene-circuit therapy, engineered therapeutics

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***This paper is dedicated to Professor Wacław Tadeusz Szybalski on the 100th anniversary of his birth**

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Abbreviations: ER, expression regulator; RN, regulatory network; TF, transcription factor; SGC, synthetic gene circuit; SHS, safe harbor site; PDX1, the pancreatic duodenal homeobox protein; NGN-3, the neurogenin; MAFA, the V-maf musculoaponeurotic fibrosarcoma oncogene homologue A; RKIP, Raf kinase inhibitory protein; BACH1, BTB And CNC Homology 1; CAR, chimeric antigen receptor; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas9, CRISPR-associated protein 9

INTRODUCTION

Since Professor Wacław Szybalski and others pioneered stable gene transfer into mammalian cells (Szybalska & Szybalski, 1962; Cepko *et al.*, 1984; Neufeld *et al.*, 1972), their efforts have been extended in many ways. Within the last six decades gene and cell therapy have reached significant milestones due to refined viral and non-viral nucleic acid delivery (Dunbar *et al.*, 2018; Buck *et al.*, 2019; Lai & Wong, 2018; Lostalé-Seijo & Montenegro, 2018) and sophisticated therapeutic strategies (Dunbar *et al.*, 2018). However, ongoing challenges with complex diseases, such as neurodegeneration (Sun & Roy, 2021) or cancer (Flavahan *et al.*, 2017; Dagogo-Jack & Shaw, 2018), cry out for even more elegant, precise and quantitative treatment solutions.

How can we proceed to meet such superior demands? We should start by rephrasing how we describe biological systems. Let us consider cellular processes from the engineering perspective and postulate several hypotheses. First, protein and RNA synthesis in each cell are governed by a complex, dynamic and modular network of mutually-controlled expression regulators (ER), i.e. transcription factors (TFs), microRNAs, etc. (Fig. 1A). Second, this regulatory network (RN) controls metabolic processes through products of regulated effector genes. Third, the global condition of the RN, delineated by protein levels stemming from rate constants, accessible TF-binding sites *etc.*, impacts the health and disease of cells (Fig. 1A and 1B.I) and tissues. Finally, its dynamic, hyperlinked structure enables the RN to act as a cellular processing core responsible for receiving and integrating signals, propagating them outside to other cells or "making decisions" (Fig. 1A) (Balázsi *et al.*, 2011). To illustrate the importance of these abstract concepts we will follow the differentiation stages of pancreatic β-cells.

NETWORK DYNAMICS IN ACTION - DIFFERENTIATION OF PANCREATIC β-CELLS

Found in pancreatic islets, β-cells are the endocrine cells responsible for synthesis and secretion of insulin, which controls the glucose level in the blood. Pathological autoimmune depletion of β-cells leads to insufficient insulin release and hyperglycemia in type 1 diabetes. Differentiated β-cell transplants can potentially cure type 1 diabetes and relieve patients from lifelong monitoring of glucose levels and insulin injections (Weir *et al.*, 2011). However, differentiation of therapeutic, insulin-producing and glucose-sensitive β-cells *in vitro* is far from being simple. Both *in vivo* and *in vitro*, β-cell differentiation is governed by three master transcription factors: (1) the pancreatic duodenal homeobox protein PDX1, (2) the neurogenin NGN-3 and (3) the V-maf musculoaponeurotic fibrosarcoma oncogene homologue A, MAFA. Relative levels of these TFs induce vast changes in the gene expression profile and the phenotypic state of differentiating cells. Importantly, the effective transition of endoderm progenitor cells into fully mature, glucose-responsive and insulin-producing β-cells requires a well-defined temporal pattern of PDX1, NGN-3 and MAFA expression. First, an increase in PDX1 levels correlates with the transition of endoderm cells into pancreatic progenitors. Subsequently, PDX1 expression must decrease simultaneously with the upregulation of NGN-1 for pancreatic progenitors to enter the endocrine progenitor stage. Finally, a secondary increase in PDX1 levels coupled with MAFA upregulation and NGN-1 decline shifts

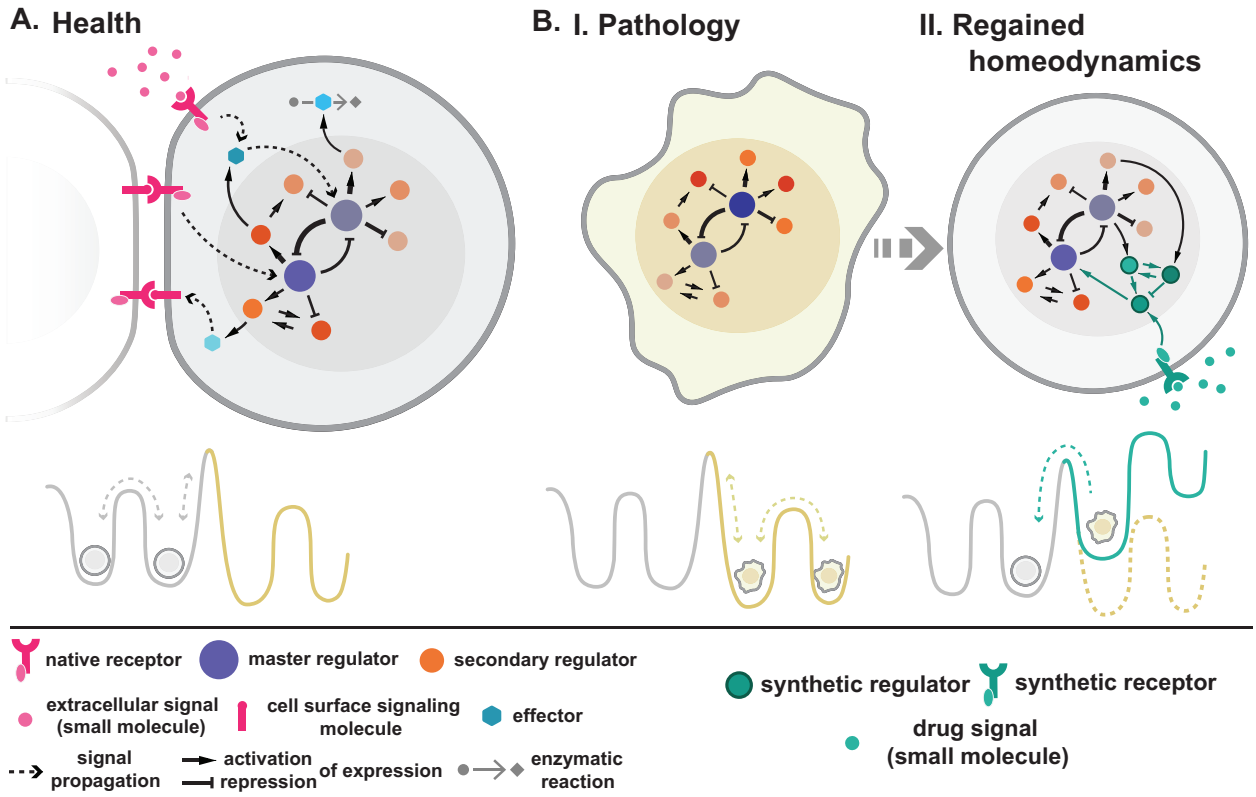

Figure 1. The dynamic regulatory network (RN) constitutes a processing core, driving cell functions.
(**A.**) Master regulators (*i.e.* transcription factors) controlling expression of multiple functional genes constitute network hubs. The protein expression levels together with reaction rates and network motifs define the global state of the RN that underlies the physiological cell states (represented as valleys in the landscape). The dynamic nature of RN function allows cells to sense internal and external signals, process them, communicate with other cells and shift into other global states according to the circumstances. (**B.**) I. If the cell enters a state beyond its homeodynamic capabilities (yellow region of the landscape) then it develops pathological behaviors and cannot adopt normally to external and internal cues. II. A synthetic gene circuit, with defined input, output and regulation points, can be incorporated into the native RN structure. When integrated efficiently, such a gene circuit can sense the cues characteristic to pathological states, integrate them and precisely tune crucial elements within native RN. Such changes bring the global expression profile back to its normal state.

endocrine progenitors into mature β-cells (Pagliuca *et al.*, 2014; Saxena *et al.*, 2016a; Habener *et al.*, 2005). In the healthy pancreas, precise timing of these changes directs global gene expression profiles and drives the cell along a defined trajectory of sequential differentiation stages required to reach the fully mature, insulin-producing and glucose-sensitive β-cell state. However, governing the same precise cascade of events *in vitro* by applying growth factors and hormones without direct TF control is particularly difficult (Pagliuca *et al.*, 2014), highlighting the need for alternate approaches.

DISRUPTION IN THE NETWORK – PATHOLOGY DEVELOPMENT

Precise control over levels of expression regulators is critical, but what happens when it is compromised? The badly timed activation of ERs and their disproportional or noisy expression can cause various cell types to enter pathological states beyond their normal capabilities to maintain homeodynamics. For instance, imbalance between the levels of Raf kinase inhibitory protein (RKIP) and metastasis activator BTB And CNC Homology 1 (BACH1) can lead to metastatic transitions in cancer due to increased expression noise and cell heterogeneity (Lee *et al.*, 2014; Gómez Tejeda Zañudo *et al.*, 2019). Likewise, disrupted levels of chromatin regulators may lead to exceedingly restrictive or permissive epigenetic landscapes, consequently increasing epigenetic instability and stochastic oncogene activation (Flavahan *et al.*, 2017). Efficient therapeutic effect requires a precise counterbalance to such pathological disruptions to bring expression profiles back to their normal physiological state (Figure 1B.II). Yet, conventional approaches of gene therapy face substantial limitations in this respect.

STRUGGLES OF GENE AND CELL THERAPY

Gene transfer lies at the foundation of gene therapy. Successful non-viral gene transfer depends on pharmacokinetics and cellular uptake of delivery agents followed by release of encapsulated nucleic acids into cells. These processes are inherently stochastic and contribute to the high heterogeneity of responses within the targeted cell population (Leonhardt *et al.*, 2014; Schwake *et al.*, 2010; Ligon *et al.*, 2014). Similarly, viral delivery and natural viral infection involve substantial cell-to-cell variation (Snijder *et al.*, 2009; Zhu *et al.*, 2020; Mikkola *et al.*, 2000; Brandt *et al.*, 2020; Russell *et al.*, 2018). Regardless of these limitations, even ideal, fully controlled gene transfer would allow for overexpression of only a few transgenes with limited command over the exact levels and kinetics of the expressed proteins. Such shortcomings are unacceptable and potentially harmful upon treatments requiring a narrow therapeutic window, precise kinetic control (Del Vecchio *et al.*, 2017) or those that

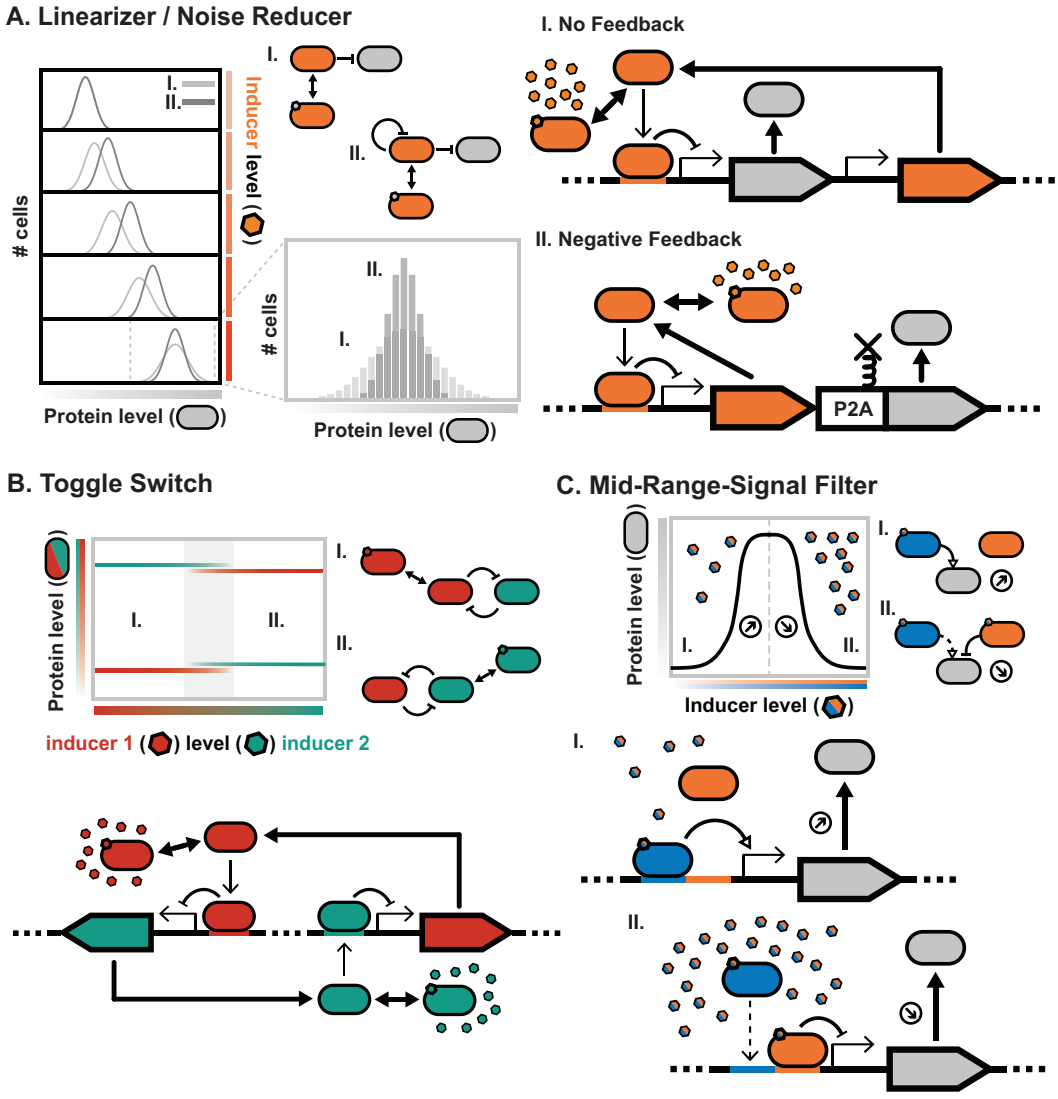

Figure 2. Examples of synthetic circuit geometries.
(**A.**) Negative autoregulation transcriptionally coupled with an effector gene (II.) decreases the expression heterogeneity and linearizes the does-response within the cell population when compared to a dual-promoter no-feedback system (I.). The bicistronic gene in II. contains a sequence of self-cleaving linker peptide (P2A) allowing effector and regulator to split shortly after translation. (**B.**) The dynamics of two mutual, signal-dependent repressors allows bistability, with one or two attractors dependent on the levels of signals deactivating each of the repressors. Such bistability works like a toggle switch where the increase in one antagonistic signals trigger the corresponding stable state (I. Green signal prevails, II. Red signal prevails. The grey area represents the range of inducer levels where both stable states coexist).C. The same molecular signal activates two transcription factors, a repressor and an activator, that regulate the same effector gene. The activator has high sensitivity to the signal and low affinity to the target, causing an increase in effector concentration at low signal levels (I.). Contrarily, the low-signal-sensitivity and high-target-affinity repressor dominates over the activator and causes a decrease in effector concentration at high signal levels (II.). Such a system allows only intermediate-level signals to propagate through and constitutes a mid-range signal filter.

involve engineered therapeutic cells. For instance, T-cell immunotherapy can cause adverse effects, such as cytokine release syndrome (Lim & June, 2017; Fitzgerald *et al.*, 2017) due to disproportionate activation of modified T cells.

SYNTHETIC BIOLOGY – BIOMOLECULAR TOOLS

The advent of synthetic biology opened new perspectives for improved gene and cell therapy. Advances in genetic and biomolecular engineering allow the design of new, synthetic biomolecules that receive and transmit signals within living cells, triggering controlled gene expression and metabolic reactions. Newly developed functional classes of biomolecules and their fragments range from chimeric transcription factors (Garg *et al.*, 2012; Thakore *et al.*, 2016; Bashor *et al.*, 2019), synthetic receptors (Morsut *et al.*, 2016; Porter *et al.*, 2011), RNA- (Culler *et al.*, 2010; Paek *et al.*, 2015; Green *et al.*, 2014), CRISPR-Cas- (Thakore *et al.*, 2016; Esvelt *et al.*, 2013) and light-induction-based molecular switches (Guinn & Balázsi, 2019; Müller *et al.*, 2015; Strickland *et al.*, 2010), localization (Spiltoir *et al.*, 2016; Niopek *et al.*, 2016, 2014) and stability (Finley, 2009; Bonger *et al.*, 2014) control factors among others (Bugaj *et al.*, 2013; Mishra *et al.*, 2014; Fux *et al.*, 2021). A prominent example of synthetic biomolecules in action is within chimeric antigen receptor (CAR)-engineered T cells (Wu *et al.*, 2020). In general, CARs constitute modified T cell receptors (TCR), where the engineered variable domain of a hapten-specific antibody replaces that of the TCR-α and/or

-β chains (Goverman *et al*., 1990; Gross *et al*., 1989), enabling cancer cell targeting. Such solutions exploit antigen specificity to recognize cancer cells, thus extending the spectrum of T cell activation beyond the capabilities of major histocompatibility complex (MHC). Development of CARs was a breakthrough in efficient T cell immunotherapy that is currently in clinical practice (Maude *et al*., 2015; Holzinger *et al*., 2016; Lundh *et al*., 2020; Lanitis *et al*., 2020). However, even these advanced engineered therapeutics suffer from the adverse effects of transgene overexpression, cell-cell variability, and the lack of contextual control of expression levels.

SYNTHETIC BIOLOGY – NEW ADAPTIVE CELL FUNCTIONS THROUGH SYNTHETIC GENE CIRCUITS

Recent endeavors enabled new directions towards precise and adaptive therapeutics. Largely, synthetic biomolecules are becoming highly modular and amenable to assemble into higher-order functional systems. Such systems, comprising signal transducers, receptors, mutually- and self-controlled ERs and their effectors, constitute synthetic gene circuits (SGC) with well-defined input, output and regulation characteristics. When integrated into the genome, SGCs interface with the native regulatory network at defined junction points and provide new sense-and-response capabilities to living cells. These new functions can be tuned by activating transgenic expression adaptively, only in specific cell types and cell states or in dose-responsive manner, hence with precise quantitative control. Importantly, the functions of SGCs and their building blocks are becoming more orthogonal relative to each other and the native cellular apparatus (Briner *et al*., 2014; Garg *et al*., 2012; Esvelt *et al*., 2013; Thakore *et al*., 2016; Green *et al*., 2014; Roybal *et al*., 2016; Stanton *et al*., 2014; Szenk *et al*., 2020), potentially reducing undesired side effects. Moreover, computer-aided design and mathematical modeling of reaction kinetics allow researchers to predict and fine-tune the functions of SGCs. Likewise, analyzing the local kinetics and the global structure of the native RN can reveal optimal control points to maximize therapeutic effect of SGCs (Gómez Tejeda Zañudo *et al*., 2019; Zañudo *et al*., 2017). Precisely-defined and complex SGC functions include Boolean logic gates (Leisner *et al*., 2010), gene oscillators (Stricker *et al*., 2008; Toettcher *et al*., 2010; Elowitz & Leibier, 2000), controllable memory buffers (Weber *et al*., 2007; Ajo-Franklin *et al*., 2007), counters (Friedland *et al*., 2009), and spatiotemporal pattern generators (Cao *et al*., 2016). Signal-sensitive, self-repressing TFs reduce the expression noise of co-expressed effector proteins, lead to linear dose-response and more uniform expression profiles within cell populations when compared to their non-self-regulated equivalents (Fig. 2A) (Guinn & Balázsi, 2019; Nevozhay *et al*., 2009; Nevozhay *et al*., 2013). Two signal-dependent, mutually repressing TFs form a toggle switch that could serve as a bistable long-term memory unit (Fig. 2B) (Kramer *et al*., 2004; Gardner *et al*., 2000).

GENE-CIRCUIT THERAPY – PROOF-OF-PRINCIPLE

Many of these and other gene circuit designs have already demonstrated their practical utility. For instance, combining two antagonistic gene regulators of different target affinity and signal sensitivity (high sensitivity, low affinity activator and low sensitivity high affinity repressor) acting on the same effector gene constitutes a mid-range-signal filter (Fig. 2C) (Greber & Fussenegger, 2010). A lineage-control gene circuit based on a vanillic-acid-driven mid-range-signal filter succeeded in driving pancreatic β-cell differentiation by controlling the desired cascade of PDX1, NGN3 and MAFA expression. Transient transfection of human induced pluripotent stem cells (hIPSCs) with the lineage-control circuit yielded mature beta-cell differentiation with unparalleled efficiency, largely exceeding the abilities of prior methods (Saxena *et al*., 2016a). On-switches responding to low molecular weight drugs combined with CARs enable tunable, spatiotemporal control over activation of engineered T-cells in attempts to restrain the adverse effects of gene overexpression on CAR T-cell based immunotherapy (Wu *et al*., 2015). Moreover, CAR-T cell therapy can be augmented by engineering tumor cells *in situ* to gain an immunomodulatory function (Nissim *et al*., 2017). SGCs can introduce novel functions into many other engineered therapeutic cells. Grafts of engineered, gene-circuit-bearing cells were instrumental in treating mouse models of obesity, metabolic syndrome, Graves' disease and gout (Rössger *et al*., 2013; Ye *et al*., 2013; Kemmer *et al*., 2010; Saxena *et al*., 2016b).

GENE-CIRCUIT THERAPY – CHALLENGES AND HOW TO FACE THEM

While promising, synthetic gene circuits still have many obstacles to overcome on their way to the clinic. The biggest shortcomings in designing efficient gene-circuit therapy for systemic applications are adverse off-target effects. Like for conventional gene therapy, effective strategies require specific targeting into the cells of interest, avoiding cytotoxic effects and adverse immune system activation. Most importantly however, SGCs need to be incorporated into safe genomic loci that facilitate full functionality and avoid any unwanted, potentially oncogenic genome alterations (Markstein *et al*., 2008; Liebert & Ellis, 2005; Russell & Grompe, 2015; Bestor, 2000). All these considerations demand precise and selective delivery and integration strategies. The CRISPR-Cas9 system offers a tempting alternative over semi-random viral genome editing. Programmable integration into predefined chromosome regions, improved specificity (Briner *et al*., 2014; Kocak *et al*., 2019) and ever-expanding modes of genome editing by different classes of CRISPR-Cas9-derived editing agents (Anzalone *et al*., 2020) are the main advantages of the system. However, reports on off-target insertions caused by CRISPR-Cas9 system and immunogenicity of CRISPR-Cas9 components remain concerning and need to be carefully addressed (Anzalone *et al*., 2020; Zhang *et al*., 2015; Dai *et al*., 2016). Independently of genome editing, systematic studies have revealed sets of human "safe harbor sites" (SHS), that are genomic regions with minimal potential for transgene deactivation and unwanted deregulation of native regulatory network upon transgene integration (Sadelain *et al*., 2012; Papapetrou & Schambach, 2016; Pellenz *et al*., 2019; Gaidukov *et al*., 2018). The exploration of the full therapeutic potential of SHSs is still ahead. Moreover, the use of orthogonal site-specific recombinases, like Flp or Cre, combined with CRISPR-Cas9 methods can further improve the site-specificity of construct insertion. For example, CRISPR-Cas9 could integrate prerequisite recognition sites of a site-specific recombinase (a so-called "landing pad") into the SHS, allowing subsequent modification through recombinase-mediated cassette exchange (RMCE) (Duportet *et al*., 2014; Ordovás

et al., 2015). Since site-specific recombinases do not create double strand breaks in chromosomal DNA and are restrictively specific to their own recognition sites, these approaches are promising to minimize off-target insertions (Grindley *et al.*, 2006; Ma *et al.*, 2014). Further advance are prospectively possible by using transient "integrator" gene circuits where the expression of CRISPR-Cas9 machinery and recombinase of choice is adjusted to achieve maximal integration efficiency without adverse side effects. Other extensions can include "safety switch" designs (Kiani *et al.*, 2015) that limit the integration only to the cells of interest, supporting current methods of targeted gene delivery (Lostalé-Seijo & Montenegro, 2018). Other approaches, such as self-replicating RNA circuits and non-integrative viral vectors, are also promising (Wagner *et al.*, 2018; Schlaeger *et al.*, 2015)

CONCLUSION

As Professor Wacław Szybalski predicted in the 1974 proceedings book "Control of Gene Expression" (Szybalski, 1974) we have entered into the era of engineered and synthetic biology. Examples and strategies presented in this review constitute the first steps in the new direction of engineered therapeutics and gene circuit therapy, sparking new anticipation and hope. For instance, the current expansion of the field of protein design and the progress in understanding of dynamic gene regulation are highly promising for future treatment strategies. Yet, there is still much to be done.

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