A retrosynthetic biology approach to therapeutics: from conception to delivery

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A retrosynthetic biology approach to therapeutics: from conception to delivery

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De novo biosynthetic pathways are designed, assembled and optimized to produce high-value compounds such as drugs and chemical building blocks from renewable resources. Microorganisms are used as synthetic platforms of systems biology where biochemical pathways are engineered into the host metabolic network. Retrosynthetic biology offers a creative pathway design concept that has gained interest because of its potential to identify novel metabolic ways for therapeutic production. Retrosynthetic biology uses the backward search of retrosynthetic analysis to devise and optimize tailor-made pathways. The retrosynthetic process can be seamlessly integrated into a complete circuitry system for therapeutic applications where production, sensing and delivery act as constitutive interconnecting parts. The aim of this review is to highlight recent efforts toward synthetic design for therapeutic development.

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Introduction

The production of high-value compounds has been largely rising for the past few years boosted by progress in metabolic engineering and synthetic biology fields. Synthetic biology aims at creating novel functional devices and systems that together with new ideas coming from the field of systems biology are enriching the toolbox of metabolic engineering for therapeutic development [1]. Metabolic engineering typically aims to identify targets for modification (e.g. enhancement of substrate range) to improve production, while synthetic biology provides the tools (e.g. genetic circuits) for construction of tailor-made cell factories and other biological devices with specific functions [2]. These advances arrive at a moment when the global demand for development of new therapeutical strategies is increasing especially for the treatment of cancer, metabolic disorders, immune diseases, as well as for infections caused by multidrug resistant pathogens [3]. This demand includes the need for new methods for the production process (economical need), the design of new drugs (with increased efficacy), and novel strategies to sense the disease (diagnostic) and to deliver the drug (*in situ* delivery) (Figure 1). For the production step, design and selection of those catalysts (i.e. enzymes) needed to produce the compound must to be addressed. To this end, chemical synthesis and biosynthesis are disciplines that have been inspired from each other. Knowledge about the structure and reaction mechanism from chemical synthesis combined with the dissection of the biochemical pathway through biochemistry, genetics and molecular biology methods enables us to improve our understanding of nature's strategies for biosynthesis and opens the space for the production of a new generation of products. Libraries of natural or synthetic compounds with improved pharmacological properties can be built by assembling new biosynthetic pathways through the recruitment of genes from a variety of organisms and craftily stringing them together. For this purpose, recent years have seen the rise of a growing trend that exploits the retrosynthesis concept in *de novo* pathway design [4••,5••,6••]. Retrosynthetic analysis, a technique having its origins in synthetic organic chemistry [7,8], conceptually starts by defining the structure and properties of the target molecule to be produced, working backward through known chemical transformations to the identification of suitable precursors. This approach applied to biology (retrosynthetic biology) is a promising tool for the development of streamlined manufacturing pipelines that can be eventually integrated with sensing and delivery circuits for advanced therapeutical applications. The process of retrosynthesis implies the identification of the involved reactions and their corresponding enzymes, and the enumeration of the biochemical pathways that can link the final product to the metabolites of the host. Furthermore, a retrosynthetic production framework should also include crucial steps such as optimization of gene expression and enzyme catalysis, which are necessary for highly efficient production pathways. Additional parts of the synthetic construction involve the rational design of ancillary circuits, a circuitry composed of control elements that can fine-tune transgene expression in response to specific conditions [9]. In that

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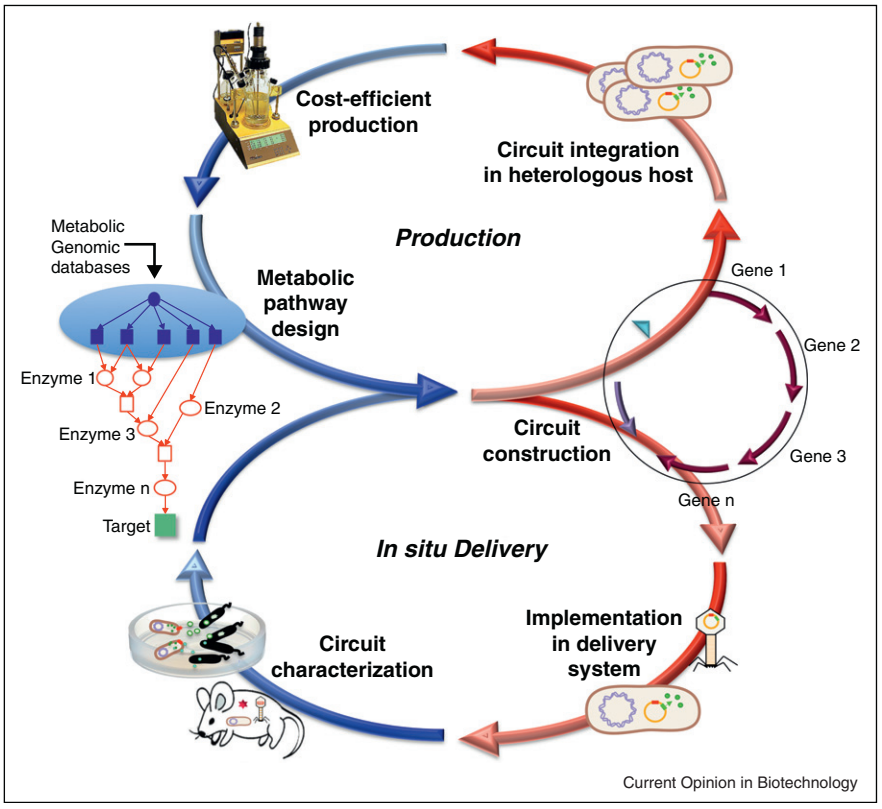
Figure 1



Therapeutic production and delivery cycles. Therapeutic production for either high-titer production or *in situ* delivery is composed of the design process, circuit construction, integration into an adequate chassis, and the optimization step. The biosynthetic pathway is designed using the retrosynthetic approach that links the target to endogenous substrates, with genomic and metabolic databases as input. Once the metabolic pathway is designed, the enzyme genes encoding the needed enzymes are assembled into an expression vector. This vector is either implemented into a production platform for high yield synthesis, or integrated into an adequate chassis for *in situ* delivery of the therapeutic. The circuit is tested *in vivo*. Feedback loops represent the continuous flow in the developments of such applications, where experimental results are fed back into the design process to improve the model, and where the optimization steps have to be tested to obtain the most efficient metabolic pathway.

sense, production, sensing and delivery work as interconnecting parts. Here we review the recent trends in producing therapeutics and their *in situ* delivery, highlighting the retrosynthetic biology related aspects emerging from them.

Microbial platform for therapeutic production

Engineering, production and functional characterization of new therapeutic candidates in microbial platforms currently represent major challenges. Increasingly, attention is focusing on low-cost production of therapeutics. The construction of such efficient heterologous pathways requires nevertheless optimizing each step of the biosynthetic pathway. To date, one of the most complete engineered pathways is the one for the production of semisynthetic artemisinin in yeast [10]. Artemisinin is one highly effective antimalarial drug, but is in short supply and unaffordable to most of the population suffering of malaria. The microbial production of this isoprenoid has been the subject of intense research starting from the original pathway implementation to successive optimizations involving strain engineering and enzyme overexpression of key reaction steps [11]. The isoprenoids are derived from the isopentenyl disphosphate (IPP) produced either from the methylerythritol phosphate (MEP) pathway or the mevalonate pathway. Moreover, the efficiency of the heterologous pathway relies often on the precursor supply. This was illustrated for the production of the isoprenoid taxadiene, which is a precursor of the potent anticancer drug taxol. The taxadiene biosynthetic route that was implemented in *Escherichia coli* was optimized using an approach that partitioned the metabolic pathway into two modules: the native MEP pathway and the heterologous terpenoid-forming pathway [12]. The taxadiene production (1 g L^{-1}) was maximized using a multivariate-modular pathway engineering that allowed to unlock *E. coli* MEP pathway in order to increase the supply of key precursor (IPP). Farnesol is yet another natural product of therapeutical interest due to its ability to diminish antifungal resistance

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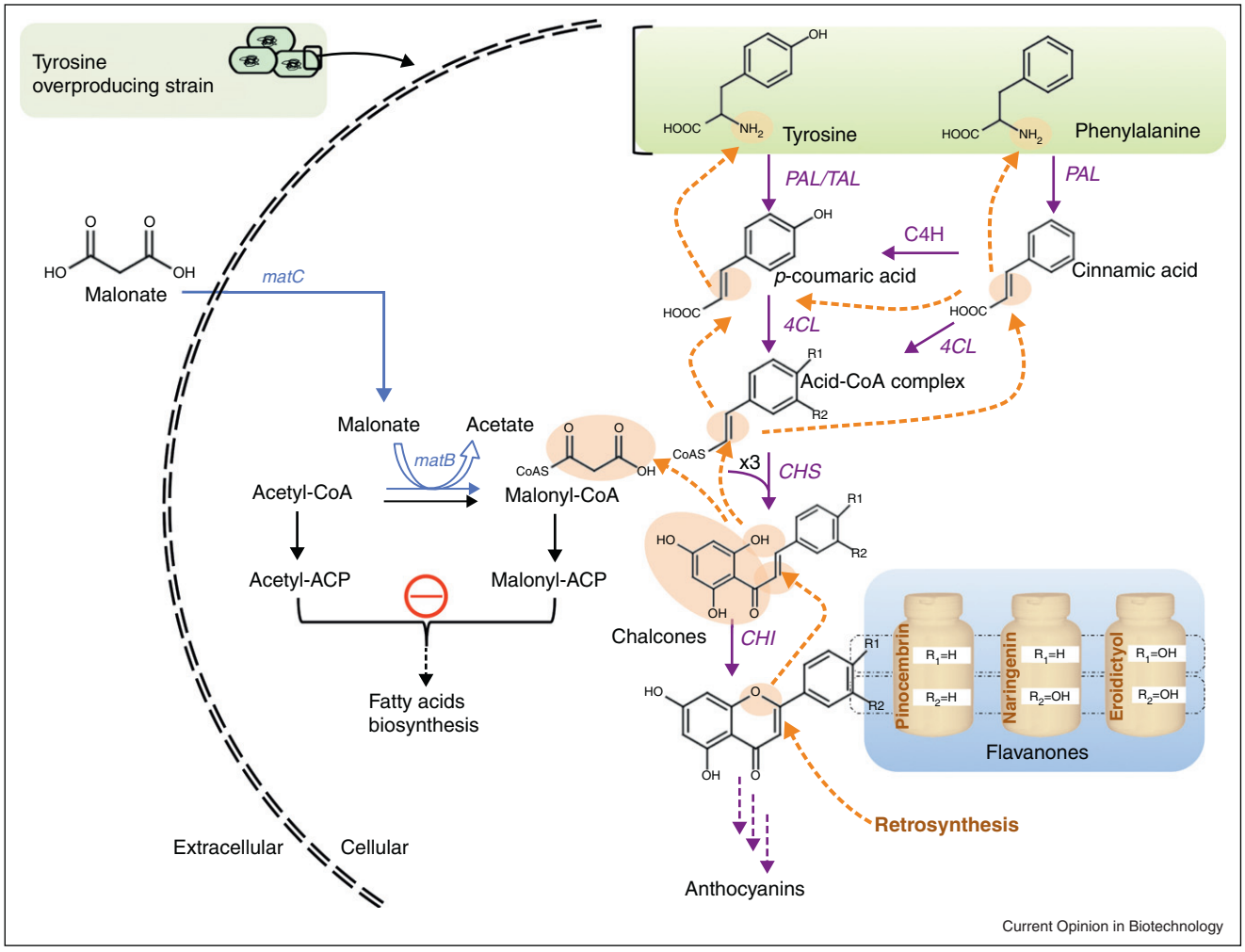
Figure 2



Engineering strategies for therapeutic production. Biosynthetic efficiency can be limited by the host cellular properties, such as precursor availability and product tolerance. Heterologous biosynthetic pathways for flavonoids in *E. coli* can be found through a retrosynthetic analysis of the enzymatic transformations, which includes phenylalanine/tyrosine ammonia lyase (PAL/TAL), 4-coumaroyl-CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI). The major bottleneck to high-level production of flavonoids is the requirement of three molecules of malonyl-CoA due to its low basal level in *E. coli*. To increase the pool of this precursor, the use of a malonate assimilation pathway from *Rhizobium trifolii* (matB, matC) allows the transport of supplemented malonate into the cell and its subsequent conversion to malonyl-CoA. Additionally, the inhibition of the fatty acid pathway increases its availability for the flavonoid pathway [17•]. With this strategy numerous plant-specific flavanones and anthocyanins can be efficiently produced from *E. coli* for pharmaceutical applications.

in *Candida albicans*, an opportunistic fungus that may cause diseases ranging from superficial mucosal infection to life-threatening systemic disorders [13]. The use of farnesol to disrupt biofilm formation has also been reported for Staphylococcus epidermis, which is the most frequent cause of nosocomial sepsis [14]. As well, farnesol seems to act as an inhibitor of tumorigenesis in several animal models [15]. The microbial production of farnesol, which is formed from its precursors IPP and farnesyl pyrophosphate, was recently developed [16]. To maximize farnesol production, *E. coli* was engineered to overexpress the farnesyl pyrophosphate synthase and to utilize an exogenous mevalonate pathway for efficient precursor synthesis. In this study, a farnesol production of 135.5 mg L^{-1} was reported, a 350-fold increase from non-optimized *E. coli*. Many other plant secondary metabolites are also natural products representing a source of promising therapeutics; for instance, purported health-promoting effects of the flavonoids have recently motivated the development of their efficient microbial production (Figure 2). Stephanopoulos and colleagues succeeded to assemble a four-enzyme heterologous pathway to synthesize high yield of naringenin from glucose after determining optimum metabolic balance in *E. coli* [17•]. The construction of this biosynthetic pathway required the assembly of genes from different organisms.

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Furthermore, a microbial production platform that produces plant alkaloids using a tailor-made biosynthetic pathway was developed [18•]. A promising application is the fermentative production of the alkaloid (*S*)-reticuline in *E. coli* without any addition of expensive precursor chemicals, with yield of 46 mg L^{-1} of product. This system would enable low-cost production of pharmaceutically important alkaloids such as the benzylisoquinoline alkaloids including analgesic compounds morphine and codeine, and antibacterial berberine and palmatine.

Biosynthetic pathway construction for therapeutic production

Integration of heterologous pathways for the biosynthesis of promising therapeutics (such as the ones cited in section 'Microbial platform for therapeutic production') into a microbial platform requires of a considerable effort in analysis, design, assembly and optimization of the route leading to the product of interest. The biosynthetic pathways are often multi-step processes involving many enzymes whose activities have to be controlled for optimal drug synthesis. To this end, computer-assisted retrosynthetic design approaches have been recently proposed. They perform an efficient search of potential pathways through the use of algorithms that iteratively import biosynthetic modules into a chassis organism until a suitable set of endogenous precursors is found [4••,5••,6••]. The power of computer-assisted retrosynthesis for synthetic biology resides in using representations of chemical biotransformations in a way that allows the description of important chemical features. This representation is based on sets of reaction rules, as those derived from bond-electron matrices [4••], or on the smallest molecular substructure that changes in the transformation [6••]. The combinatorial complexity associated with such highly generative representations is a major issue that our group has recently addressed by proposing a tradeoff solution based on molecular signatures [5••]. This method can control the complexity of the pathway search through the selection of the level of specificity in the reaction representation. The construction of the complete identified therapeutic biosynthetic pathways into a chassis requires advanced cloning techniques with standardized methods. Standardization has the great advantage to offer the possibility of finding the best gene combination for pathway efficiency. To this end, a library of standardized expression vectors has been built in which origins of replication, inducible promoters, and antibiotic resistance genes have been combined [19]. In addition, available quantitative datasheets for the standardized expression vectors make the predictability of gene expression possible when multiple plasmids are needed. The need for standardization in therapeutic synthesis is also addressed in the retrosynthetic design through the characterization of the different biosynthetic modules that form the pathway. Many aspects are to be considered, such as thermodynamics feasibility, organism compatibility for expression [20], toxicity of metabolites [21], or predictability of enzymatic activity [22]. Overall, individual performances for the predicted pathways need to be characterized in order to prioritize the engineering of the most promising routes among all possible enumerated pathways into the chassis organism [23]. In retrosynthetic pathway optimization, the general objective is to produce the desired therapeutic compound with high production rates while retaining the cell ability to sustain growth and survival. Engineered organisms however produce therapeutics through pathways composed of heterologous enzymes that might not function together in nature. Therefore, strains need to be optimized to cope with the flux imbalance problem toward the overproduction of the target. To this end, several metabolic engineering strategies have been recently proposed in order to determine the optimal set of metabolic modifications through the use of elementary modes [24], flux balance analysis [25], or mixed-integer linear programming [26]. Recent developments have also addressed the control of microbial electrosynthesis to alleviate redox imbalances during the synthesis of the target compounds [27,28]. Additionally, enzymes involved in the designed synthetic pathways might be subject to protein engineering and directed evolution for the improvement of enzyme activity and stability and for the alteration of substrate specificity and kinetic characteristics [29,30,31]. Moreover, biosynthetic design enables hierarchical modularization of the parts in the metabolic pathway as for instance in the use of modular scaffolds for the artemisinin pathway [11], or in the multivariate optimization of upstream/downstream modules for the taxol pathway [12]. In a similar fashion, retrosynthetic design can be applied to the production of precursors in natural biosynthetic modules, such as polyketide synthases [32], or nonribosomal peptide synthases [29,33]. The use and reuse of those modules allow the construction of cell-based biosynthetic systems for a plethora of innovative therapeutic applications going from synthesis to environmental sensing [34,35].

Circuits for therapeutic production and *in situ* delivery

The retrosynthetic-based design pathway for drug production is an essential part of the circuitry that can be adapted for industrial production or *in situ* delivery of drugs (Figure 1). The circuitry is important to ensure the energy balance in the cell to maintain its growth and viability especially when toxic elements are involved. Circuits can also be designed to control the targeting and the release of the therapeutic (Table 1). Within this context, devices for sensing pathogenic conditions (e.g. cancer cells, pathogenic microorganisms, metabolic states) and fine-tuning transgene expression in response were developed [36–38]. The variety of the sensing tools extends from small molecules as autoinducers to light sensitive devices [39] and miRNA detection systems [40]. These devices have demonstrated their potential to become adequate carriers for bio-retrosynthetic-based

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Table 1

Synthetic biology devices with therapeutic application

Therapeutic application	Engineered chassis	Sensing device	Production device	Delivery device	Mode of action	Animal model	References
Bacterial infection	Bacteriophage M13	–	LexA3 protease, SoxR protein, CsrA protein, OmpF porin	–	Overexpression of LexA3 protease, SoxR, CsrA or OmpF porin enhances bacterial susceptibility to antibiotics	Mouse	[44]
Bacterial infection	–	Antibody	*E. coli* S-adenosylhomocysteine nucleosidase and S-ribosylhomocysteinase	–	The antibody targets the site of interest and the enzymes convert the locally available precursors into autoinducer-2 quorum-sensing molecule, thus modulating bacterial population.	–	[51,52]
Bacterial infection	*E. coli* Nissle	–	*Vibrio cholera* pyridoxal phosphate-dependent acyl-CoA transferase	–	*V. cholera* pyridoxal phosphate-dependent acyl-CoA transferase is the last enzyme of the quorum-sensing molecule CAI-1 synthesis. *In situ* production of CAI-1 by the engineered *E. coli* Nissle perturbed the quorum-sensing dependent virulence of *V. cholera* in infant mice gut leading to increased survival.	Mouse	[50]
Bacterial infection	*E. coli*	*Pseudomonas aeruginosa* quorum-sensing LuxR protein	Pyocin S5	E7 lysis protein	Upon detection of the quorum-sensing signal of *P. aeruginosa*, pyocin S5 and E7 lysis protein are expressed. E7 lysis protein destroys *E. coli* thus releasing pyocin S5 that kills *P. aeruginosa*	–	[42•]
Dengue fever	*Aedes aegypti*	Sex-specific alternative splicing	Intron-containing synthetic tetracycline-repressible transactivator (tTA)	–	The dengue vector *Aedes aegypti* was engineered to carry a synthetic tTA containing an intron that is spliced out in a female-specific manner leading to tTA expression and thus to a flightless phenotype and finally to death.	Mosquito	[53]
Malaria	*Anopheles gambiae*	Sex-specific promoter	Homing endonuclease *I-SceI*	–	The homing endonuclease is expressed under the control of a male germline promoter and disseminates into the entire mosquito population.	Mosquito	[54]
Cancer	*Salmonella enterica*	*Pseudomonas putida* salicylate-controlled promoter	Cytosine deaminase		Upon acetyl salicylic acid oral administration (rapidly converted into salycilate), the engineered *S. enterica* expresses the cytosine deaminase that converts 5-fluorocytosine into the cytostatic 5-fluorouracil	Mouse	[46]
Cancer	Human embryonic kidney (HEK-293) cells	RNA aptamers	*Herpex simplex* virus type-1 thymidine kinase	–	Upon detection of high concentrations of tumor proteins, the expressed thymidine kinase converts nucleotide analogs into toxic compounds that destroy cancer cells	–	[55]

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Table 1 (*Continued*)

Therapeutic application	Engineered chassis	Sensing device	Production device	Delivery device	Mode of action	Animal model	References
Cancer	Various human cancer cells	Inflammatory chemokine CXCL1, synovial sarcoma X-breakpoint protein-1 SSX1, histone-H2A1 promoters	*Herpex simplex* virus type-1 thymidine kinase	–	Upon simultaneous detection of two different tumor signals, the expressed thymidine kinase converts nucleotide analogs into toxic compounds that destroy cancer cells	–	[56]
Cancer	Human cervical adenocarcinoma cells (HeLa)	miRNA	Human Bcl-2-associated X protein	–	When the expression level of several endogenous miRNAs matches a predetermined reference profile characteristic of a cancer cell, apoptosis is triggered by production of human Bcl-2-associated X protein	–	[40]
Cancer	*Salmonella enterica* Typhimurium	*E. coli* anaerobic-inducible nirB promoter	Murine TNF-related apoptosis-inducing ligand (TRAIL)		In the hypoxic environment of tumors, the TRAIL protein was expressed and secreted by the engineered *S. enterica* Typhimurium thus leading to apoptosis of cancer cells	Mouse	[45]
T-cell therapy	T cells	Theophylline-responsive ribozyme switches	γ-Chain cytokine IL-15	–	Upon theophylline administration, the γ-chain cytokine IL-15 was expressed thus modulating T-cell growth rate	Mouse	[57]
Tumor lysis syndrome and Gout	Human cervical adenocarcinoma cells (HeLa)	*Deinococcus radiodurans* urate receptor	*Aspergillus flavus* urate oxidase	Protein secretion tag	At high urate concentration, the expressed urate oxidase metabolize uric acid	Mouse	[41•]
Inflammatory bowel disease	*Bacteroides ovatus*	Xylan-controlled xylanase promoter	Human keratinocyte growth factor-2 Human transforming growth factor-β	Protein secretion tag	Upon xylan oral administration, the KGF-2 or TGF-β is expressed by the engineered *B. ovatus* thus leading to significant clinical improvement of colitis	Mouse	[49]
Oral mucositis	*Lactococcus lactis*	–	Human Trefoil Factor 1	Protein secretion tag	The engineered *L. lactis* secrets the Trefoil Factor 1, an essential protein in restitution and repair of gastrointestinal tract epithelial cells	Hamster, rat	[48]
Diabetes	*E. coli* Nissle	Glucose-responsive promoter	Glucagon-like peptide-1 and pancreatic and duodenal homeobox gene 1	Protein secretion tag	The proteins produced by the engineered *E. coli* stimulate epithelial cells to produce insulin	–	[47]
Diabetes	Human embryonic kidney (HEK-293) cells	Melanopsin	Glucagon-like peptide-1	Protein secretion tag	Upon blue light illumination, a synthetic transduction cascade is induced and leads to expression of glucagon-like peptide-1, thus modulating glucose homeostasis	Mouse	[39]
Artificial insemination	Human embryonic kidney cells (HEK-293)	Luteinizing hormone receptor	–	*B. subtilis* cellulase	Upon luteinizing hormone surge, the cellulose-sulfate capsule is degraded by the expressed cellulase thus liberating the spermatozoids	Cow	[58]

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designed pathways enabling thus a controlled *in situ* drug production. One such a refined circuit was developed for treating metabolic disorders like hyperuricemia associated with the tumor lysis syndrome and gout [41•]. To this end, the restoration of the urate homeostasis in mice was shown using HeLa cells engineered to sense high urate concentrations via a urate receptor (from *Deinococcus radiodurans*) and to express the urate oxidase (from *Aspergillus flavus*) that upon excretion metabolizes uric acid. This type of closed-loop control of transgene expression depending only on substrate concentration illustrates a promising design principle of automated and independent devices that can adjust their response in a therapeutically efficient manner. Recent examples of elaborated circuits include also controlled delivery devices. It is the case of an engineered *E. coli* system where upon detection of the quorum-sensing signal of *Pseudomonas aeruginosa*, two toxic proteins are expressed in the chassis: pyocin S5 and E7 lysis protein. The E7 lysis protein targets and destroys the chassis *E. coli* cells thus releasing pyocin S5 that kills *P. aeruginosa* [42•]. This suicide type of delivery systems copes with issues relating to secretion of the therapeutic out of the producing cell and with safety regarding the dissemination of the vector.

Conclusion

By integrating information from both metabolic networks and biochemical transformations, the retrosynthetic biology approach is at the intersection of top-down systems biology and bottom-up synthetic biology [42•,43]. As such, it brings together in a consistent way the four conceptual steps for process synthesis engineering: abstraction, modularization, standardization, and optimization [43]. Moreover, the retrosynthetic biology has the potential of providing hypothesis on novel metabolic pathways. In that sense, two modes of action can be proposed: from sequence data, new biosynthetic genes can be identified and the retrosynthesis process can thus propose hypothesis on product formation from those genes, highlighting new metabolic pathways. Conversely, from the products synthesized, the retrosynthesis process can allow the identification of biosynthetic genes and thus reconstruct the pathway. The development of affordable sequencing techniques, together with progress in genome annotation, is dramatically increasing the amount of data that can potentially be provided to retrosynthetic biology design process. Promisingly, the retrosynthetic biology addresses the increasing demand for drug development by proposing effective methodologies to rationalize the design and implementation processes. Biological parts and tailor-made devices can be integrated by these means into the design of novel therapeutic circuits. Further developments would lead to customized therapies designed for a patient's physiology, contributing to novel applications in the emerging discipline of personalized medicine. Specificity and targeting are promising strategies brought with the use of bacteriophage for bacterial infection [44], or with the use of programmed bacteria that can target specific cancer-related pathways once inside the cells (a strategy known as bactofection) [45,46]. Yet, another system is the use of commensal microorganisms as vectors for deploying synthetic circuits to fight the disease [42•,47–50]. The production of therapeutics can be turned-on or turned-off only when prescribed molecular interventions are needed by controlling the production of therapeutics through cell-based sensors able to detect pathological conditions. As the catalog of such biosynthetic, sensing, and regulation circuits will become more widespread; we envision a bright development of robust and adaptive controlled devices for efficient industrial production and *in situ* delivery of therapeutics.

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- •• of outstanding interest

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