

A synthetic free fatty acid-regulated transgene switch in mammalian cells and mice

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ABSTRACT

Trigger-inducible transgene expression systems are utilized in biopharmaceutical manufacturing and also to enable controlled release of therapeutic agents *in vivo*. We considered that free fatty acids (FFAs), which are dietary components, signaling molecules and important biomarkers, would be attractive candidates as triggers for novel transgene switches with many potential applications, e.g. in future gene- and cell-based therapies. To develop such a switch, we rewired the signal pathway of human G-protein coupled receptor 40 to a chimeric promoter triggering gene expression through an increase of intracellular calcium concentration. This synthetic gene switch is responsive to physiologically relevant FFA concentrations in different mammalian cell types grown in culture or in a bioreactor, or implanted into mice. Animal recipients of microencapsulated sensor cells containing this switch exhibited significant transgene induction following consumption of dietary fat (such as Swiss cheese) or under hyperlipidaemic conditions, including obesity, diabetes and lipodystrophy.

INTRODUCTION

Synthetic trigger-controlled gene switches that enable adjustable and reversible fine-tuning of target protein production are required for functional genomic research (1), drug discovery (2), gene therapy (3), biopharmaceutical manufacturing (4) and tissue engineering (5). Already, huge advances in synthetic biology have led to the creation and engineering of artificial biological pathways, organisms and devices through reassembling catalogued and standardized biological units in a systematic, rational and predictable manner to obtain novel and useful functions (6), and a

wide range of transgene control systems with unprecedented precision and reliability has been developed. These gene switches have become essential components for the construction of sophisticated transcription/translation networks such as oscillators (7), inter-cellular communication systems (8) and biocomputers (9). Since switches that use exogenous inducers may cause secondary side effects, there is a need for gene switches that are responsive to endogenous metabolites/biomarkers, and many such systems that interface directly with host metabolism/physiology have already been validated in animal models, including models of gout (10), hyperthyroidism (11), liver injury (12) and diabetes (13).

Free fatty acids (FFAs) are important energy sources for most body tissues and key components of cell membranes and various lipid classes (triglycerides, phospholipids, cholesteryl esters) (14). They also serve as second messengers regulating cellular processes (15), precursors to various lipid mediators (16), factors influencing protein acylation (17) and modulators of gene transcription (18) and signal transduction (19). All of these activities are largely dependent on the carbon-chain lengths of FFAs. Short-chain fatty acids have less than six carbons, medium-chain fatty acids have 6–12 carbons and long-chain fatty acids have >12 carbons (20). Medium- and long-chain FFAs are primary energy sources metabolized through β -oxidation in tissues and constitute the predominant types of fatty acids in the bloodstream (21). FFA levels are predominantly regulated via nutrition and lipolysis from triglycerides, cholesterol, lipoproteins and adipose tissues, depending upon the energy demands of the body (14). Therefore, circulating FFAs are an important and sensitive biomarker of physiological status. We thus considered that FFAs would be attractive candidates as triggers for novel transgene switches.

Human G-protein coupled receptor 40 (GPR40), which is abundantly expressed in the pancreas, functions as a receptor for medium- to long-chain FFAs (22,23). Here, capitalizing on its high sensitivity and broad range of activation

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by major serum FFAs at physiologically relevant concentrations (22–24), we adopted human GPR40 as a sensor module and rewired its signal activation to transgene expression through synthetic promoters in mammalian cells. We show that the optimized FFA-activated transgene switch (FATS) thus obtained can sense and report blood fatty acid levels induced by dietary fat consumption or chronically altered physiological states. We also show that it can provide dose- and time-specific control of product gene expression in a bioreactor.

MATERIALS AND METHODS

Design of plasmids

Comprehensive design and construction details for all expression vectors are provided in Table 1.

Cell culture and transfection

Human embryonic kidney cells (HEK-293T, ATCC: CRL-11268), baby hamster kidney cells (BHK-21, ATCC: CCL-10), human cervical adenocarcinoma cells (HeLa, ATCC: CCL-2), human fibrosarcoma cells (HT-1080, ATCC: CCL-121) and human bone marrow stromal cells immortalized with human telomerase reverse transcriptase (hMSC-hTERT) (25) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Basel, Switzerland, cat. no. 52100–39) supplemented with 10% (v/v) fetal calf serum (FCS, Sigma-Aldrich, St. Louis, MO, USA, cat. no. F7524) and 1% (v/v) penicillin/streptomycin solution (Biowest, Nuaille, France; cat. no. L0022–100). Wild-type Chinese hamster ovary cells (CHO-K1, ATCC: CCL-61) were grown in ChoMaster[®] HTS (Cell Culture Technologies, Gravesano, Switzerland; cat. no. HTS-8) supplemented with 5% (v/v) FCS and 1% penicillin/streptomycin solution. FreeStyle[™]-293F suspension cells (Life Technologies, Carlsbad, CA; cat. no. R79007) were cultivated in FreeStyle[™]-293 Expression Medium (Life Technologies; cat. no. 12338018) supplemented with 1% penicillin/streptomycin solution and grown in flasks on an orbital shaker (IKA KS 260 basic; IKA-Werke GmbH, Staufen im Breisgau, Germany; cat. no. 0002980200) set to 100–150 rpm. All cell types were cultivated at 37°C in a humidified atmosphere containing 5% CO₂. Cell number and viability were quantified using an electric field multichannel cell counting device (Casy Cell Counter and Analyzer Model TT, Roche Diagnostics GmbH). For transfection of BHK-21, CHO-K1, HeLa and HT-1080, 4 × 10⁵ cells seeded per well of a six-well plate 12 h before transfection were incubated overnight with a 4:1 PEI:DNA mixture solution (PEI: polyethylenimine; MW 40 000, Polysciences, Inc., Warrington, USA; 1 mg/ml in water, pH 7.0), while a 3:1 PEI:DNA mixture was used for transfection of HEK-293T, hMSC-hTERT and FreeStyle[™]-293F cells. After transfection, cells were detached by incubation with 2 ml Trypsin–EDTA (Biowest, Nuaille, France, cat. no. L0940) for 3 min at 37°C, collected in 8 ml cell culture medium, centrifuged for 3 min at 290 × g, and re-suspended with inducer-containing DMEM (3.5 ml/well of six-well plate) for re-seeding (300 μl/well for 48-well plate; 150 μl/well for 96-well plate). All cells were cultivated in

their specific media containing different inducer concentrations, and reporter protein levels were assayed at the indicated times.

SEAP quantification

The production of SEAP (human placental-secreted alkaline phosphatase) was quantified in cell culture supernatants as described previously (26). Serum levels of SEAP were profiled using a chemiluminescence-based assay (Roche Diagnostics GmbH, Mannheim, Germany).

Inducer and reagents

GW9508 (cas. no. 885101-89-3, Sigma-Aldrich Chemie GmbH) was dissolved in DMSO to a final concentration of 25 mM as a stock solution. TAK875 (cas. no. A11018, AdooQ BioScience) was dissolved in DMSO to 12.5 mM. Palmitic acid (cas. no. 57103), *cis*-4,7,10,13,16,19-docosahexaenoic acid (cas. no. 6217545), linoleic acid (cas. no. 60333), oleic acid (cas. no. 112801), 5,8,11-eicosatrienoic acid (cas. no. 13488227), myristic acid (cas. no. 544638), stearic acid (cas. no. 57114), lauric acid (cas. no. 143077), linolenic acid (cas. no. 463401), arachidonic acid (cas. no. 506321) and rosiglitazone (cas. no. 122320–73-4) were purchased from Sigma and diluted in DMSO to obtain 50 mM stock solutions. Butter, Swiss cheese, fresh whole milk, extra virgin olive oil, sesame oil and sunflower oil were purchased from Coop, Basel. Charcoal-stripped fetal bovine serum was from Invitrogen (cas. no. 12676-011).

Animal experiments

Cell implants were produced by encapsulating pYL4/pYL1-transgenic HEK-293T cells into coherent alginate-poly-(L-lysine)-alginate beads (400 μm; 200 cells/capsule) using an Inotech Encapsulator Research Unit IE-50R (Buechi Labortechnik AG, Flawil, Switzerland) set to the following parameters: 25 mL syringe operated at a flow rate of 450 units, 200 μm nozzle with a vibration frequency of 1024 Hz and bead dispersion voltage of 1.2 kV, stirrer speed set at 4.5 units. Six-week-old male Swiss mice (oncins France souche 1, Charles River Laboratory, Lyon, France) weighing 25–28 g were intraperitoneally injected with 700 μl serum-free DMEM containing 2 × 10⁶ cells and were orally gavaged with oleic acid (300 μl; 44.5, 89 or 179 mg/mL) or Swiss cheese (300 μl, 1.00 g/ml in 4 M urea/1% SDS) and control solution (urea 4 M/1% SDS), twice daily. To prepare an HIV-associated lipodystrophy mouse model, wild-type male C57BL6 mice (The Jackson Laboratory, Maine, USA) at the age of 8 weeks were dosed via intragastric gavage twice daily with ritonavir (200 μl, 3 mg/ml) (Interchim, Code: R1519, France) or an equal volume of vehicle solution (7.8% Ethonal, 7.8% Tween 80 in water (v/v)) for 20 days. This dose corresponds to a clinically relevant concentration of the HIV protease inhibitor (30–60 mg/kg·d). Mice were fed on normal chow throughout the experiment and were fasted for 4–5 h before cell injection on the day of experiment. To prepare high fat (HF)-induced obese mice, 4-week-old male C57BL6 mice were kept in a temperature-controlled room (22 °C) on a

Table 1. Plasmids and oligonucleotides used and designed in this study

Plasmid	Description	Reference
pcDNA3.1(+)	Mammalian expression vector (P_{hCMV} -MCS-pA).	Life Technologies
pSEAP2-control	Constitutive SEAP expression vector (P_{SV40} -SEAP-pA).	Clontech
GPR40-FLAG	P_{hCMV} -driven expression vector encoding human GPR40 tagged C-terminally with FLAG epitope (P_{hCMV} -hGPR40-FLAG-pA).	(82)
pNifty-SEAP	NF- κ B inducible SEAP expression vector ($P_{NF-\kappa B-ELAM}$ -SEAP-pA).	InvivoGen
pHY30	P_{NFAT} -inducible SEAP expression vector (P_{NFAT} -SEAP-pA).	(83)
MKp37	P_{hCMV} -driven expression vector encoding TetR-Elk-1 hybrid transcription factor (P_{hCMV} -TetR-Elk-1-pA).	(84)
pMF111	TetR-responsive SEAP expression vector ($tetO_7$ - $P_{hCMVmin}$ -SEAP-pA).	(85)
pMSCV	Calcium responsive insulin expression vector ($P_{MSCV-CRE-SRE-NFAT}$ -insulin-pA).	(29)
pAT14	$P_{SRE-NFAT}$ -driven SEAP expression vector ($P_{SRE-NFAT}$ -SEAP-pA).	(86)
pKR135	Constitutive mammalian LSR expression vector (P_{hCMV} -LSR-pA).	(63)
pMG10	Vector encoding a P_{TigR1} -driven SEAP expression unit (P_{TigR1} -SEAP-pA).	(63)
pYL1	$P_{CRE-SRE-NFAT}$ -driven SEAP expression vector ($P_{CRE-SRE-NFAT}$ -SEAP-pA). $P_{CRE-SRE-NFAT}$ was PCR amplified from pMSCV using OYL11 (5'-GCCCGCTCGAGCGCACCAGACAGTGACG-3', <i>XhoI</i> underlined) and OYL12 (5'-CCCCCAAGCTTCTGGAATTCGAGCTTCCATTAT-3', <i>HindIII</i> underlined), digested with <i>XhoI/HindIII</i> and ligated into pSEAP2-control (<i>XhoI/HindIII</i>).	This work (GenBank accession no. MH594278)
pYL2	pUC57-derived vector containing 5 \times AP-1 binding site, 5 \times NF- κ B binding site followed by an IFN- β promoter.	This work
pYL3	$P_{AP-1-NF-\kappa B}$ -driven SEAP expression vector ($P_{AP-1-NF-\kappa B}$ -SEAP-pA). $P_{AP-1-NF-\kappa B}$ was excised from pYL2 using <i>XhoI/HindIII</i> and cloned into pSEAP2-control (<i>XhoI/HindIII</i>).	This work
pYL4	P_{hCMV} -driven hGPR40 expression vector (P_{hCMV} -hGPR40-pA). hGPR40 was PCR amplified from GPR40-FLAG using OYL20 (5'-CCGCGGAAGCTTCAATGAATCGCCACCATGGACCTGCCCGCAGCTCTCCTTC-3' <i>HindIII</i> underlined) and OYL21 (5'-AGCTAGGTAACTCTAGATTACTTCTGGGACTTGCCCCCTGCGT-3' <i>XbaI</i> underlined), digested with (<i>HindIII/XbaI</i>) and cloned into pcDNA3.1(+). (<i>HindIII/XbaI</i>).	This work (GenBank accession no. MH607407)
pYL5	P_{CRE} -driven SEAP expression (P_{CRE} -SEAP-pA). P_{CRE} was PCR amplified from pYL1 using OYL11 (5'-GCCCGCTCGAGCGCACCAGACAGTGACG-3', <i>XhoI</i> underlined) and OYL13 (5'-GGAGACAGATCTACCGGGTTCTCCCAT-3', <i>BglII</i> underlined), digested with (<i>XhoI/BglII</i>) and inserted into pYL1 (<i>XhoI/BglII</i>).	This work
pYL6	P_{SRE} -driven SEAP expression (P_{SRE} -SEAP-pA). P_{SRE} was PCR amplified from pYL1 using OYL14 (5'-CCGCCCTCGAGCGGGAGGATGTCCATATTA-3', <i>XhoI</i> underlined) and OYL15 (5'-CGAGGAAGATCTCGGGAGATGTCCTAATATGG-3', <i>BglII</i> underlined), digested with (<i>XhoI/BglII</i>) and inserted into pYL1 (<i>XhoI/BglII</i>).	This work
pYL7	$P_{CRE-SRE}$ -driven SEAP expression ($P_{CRE-SRE}$ -SEAP-pA). $P_{CRE-SRE}$ was PCR amplified from pYL1 using OYL11 (5'-GCCCGCTCGAGCGCACCAGACAGTGACG-3', <i>XhoI</i> underlined) and OYL12 (5'-CCCCCAAGCTTCTGGAATTCGAGCTTCCATTAT-3', <i>HindIII</i> underlined), digested with (<i>XhoI/HindIII</i>) and inserted into pYL1 (<i>XhoI/HindIII</i>).	This work

Abbreviations: P_{hCMV} , human cytomegalovirus immediate early promoter; P_{SV40} , simian virus 40 promoter; $P_{hCMVmin}$, minimal version of P_{hCMV} ; SEAP, human placental secreted alkaline phosphatase; pA, polyadenylation signal; ELAM, endothelial cell-leukocyte adhesion molecule; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NFAT, nuclear factor of activated T-cells; *tetO*, tetracycline-responsive operator; TetR, Tet repressor; MSCV, murine stem cell virus; CRE, cyclic adenosine monophosphate response element; SRE, serum response element; AP-1, activator protein 1; IFN- β , interferon beta.

12-h light-dark cycle. Upon arrival, the mice were divided into two groups and fed on either a HF diet (60 kcal% fat diet, T-58Y1-58126, TestDiet, UK) or a control diet (4 kcal% fat diet; Teklad Global 2016 diet, Envigo, France) for 15 weeks. Body weight was measured weekly. As genetically disposed diabetic animals, *db/db* mice (female, 8 weeks old) were purchased from Janvier Labs. Blood samples were collected 24 h after treatment and serum was isolated using BD Microtainer[®] SST tubes according to the manufacturer's instructions (centrifugation for 5 min at 10 000 g; Becton Dickinson, Plymouth, UK; cat. no. 365967). All experiments involving animals were performed according to the directives of the European Community Council (2010/63/EU), approved by the French Republic (project nos. DR2014-42 and DR2016-13), and carried out by Ghislaine Charpin-El Hamri (no. 69266309) and

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RESULTS

Design of an FFA-activated transgene switch (FATS) in mammalian cells

The constitutively expressed human GPR40 construct (pYL4, P_{hCMV} -hGPR40-pA) senses extracellular FFA levels and triggers an increase of intracellular calcium through a $G\alpha_{q/11}$ -dependent signaling pathway (22,23). By rewiring the intracellular calcium surge via NFAT-dependent activation (27) of a calcium-responsive promoter (pYL1, $P_{CRE-SRE-NFAT}$ -SEAP-pA) containing cyclic adenosine monophosphate response elements (CRE), serum re-

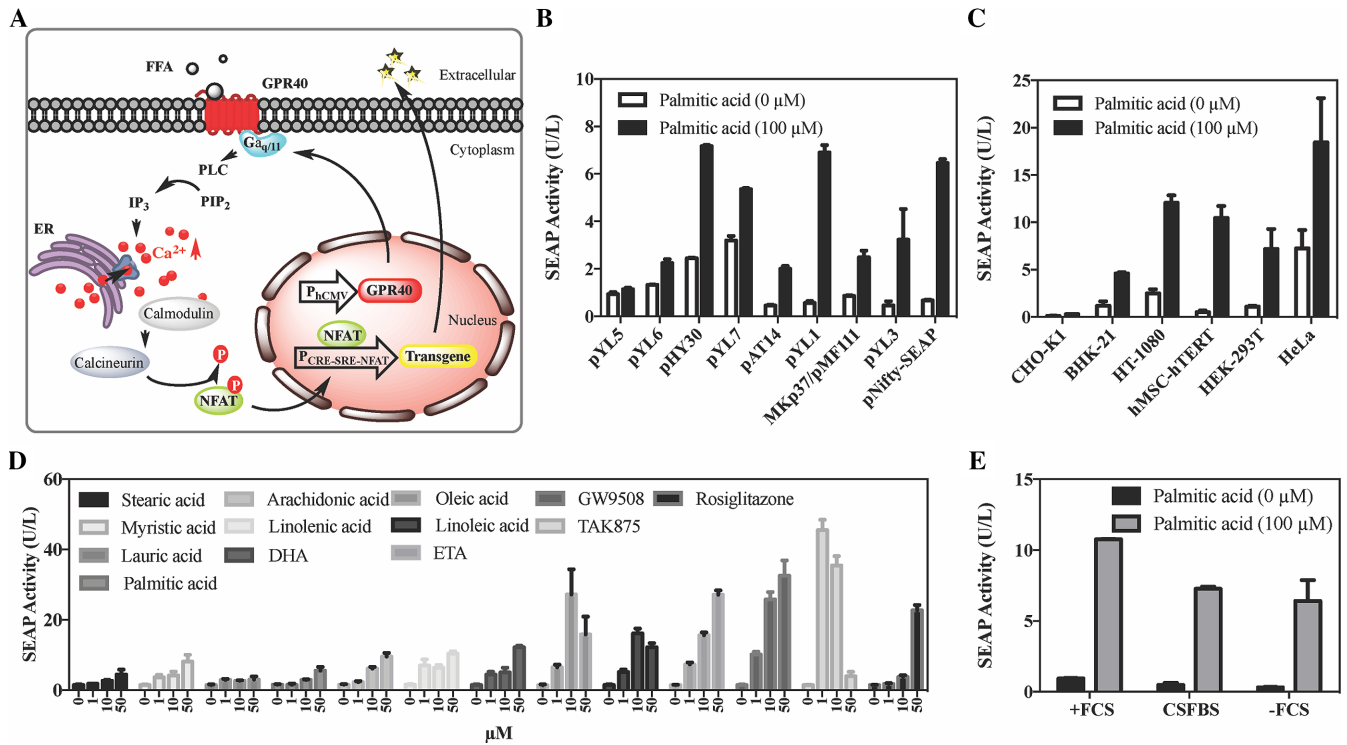


Figure 1. (A) Design of a transcription-control device activated by free fatty acids (FFAs). Interaction of human GPR40 with FFAs induces conformational changes that activate its coupled G protein subunit $G_{\alpha q/11}$, which in turn catalyzes PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol triphosphate (IP₃), leading to mobilization of endoplasmic reticulum (ER) Ca²⁺ stores. This cytoplasmic Ca²⁺ surge is sensed by calmodulin, which activates calcineurin, leading to the dephosphorylation of NFAT and translocation to the nucleus. Transgene expression is initiated through the binding of NFAT to a synthetic promoter (P_{CRE-SRE-NFAT}) composed of three Ca²⁺ response elements in *cis* arrangement: CRE, SRE and NFAT. PLC, phospholipase C; P indicates a phosphate group; CRE, cyclic adenosine monophosphate response element; SRE, serum response element; NFAT, nuclear factor of activated T cell response element. (B) Optimization of FFA-controlled SEAP expression with different promoter configurations. HEK-293T cells were co-transfected with human GPR40-encoding expression vector (pYL4; P_{hCMV}-hGPR40-pA) and an expression vector encoding SEAP under control of different promoters (pYL5, P_{CRE}-SEAP-pA; pYL6, P_{SRE}-SEAP-pA; pHY30, P_{NFAT}-SEAP-pA; pYL7, P_{CRE-SRE}-SEAP-pA; pAT14, P_{SRE-NFAT}-SEAP-pA; pYL1, P_{CRE-SRE-NFAT}-SEAP-pA; MKp37/pMF111, P_{hCMV}-TetR-Elk-1-pA/*tetO*₇-P_{hCMV}^{min}-SEAP-pA; pYL3, P_{AP-1-NF-κB}-SEAP-pA; pNifty-SEAP, P_{NF-κB-ELAM}-SEAP-pA), and cultivated for 24 h in the presence of 100 μM palmitic acid. (C) Validation of FFA-inducible SEAP expression in different mammalian cells. Cells were transfected with pYL4 (P_{hCMV}-hGPR40-pA)/pYL1 (pYL1, P_{CRE-SRE-NFAT}-SEAP-pA) and incubated with 100 μM palmitic acid for 24 h. (D) Responsiveness of FATS to different fatty acids and synthetic molecules. pYL4/pYL1-transfected HEK-293T cells were incubated with various fatty acids and chemicals at increasing concentrations and SEAP expression was assayed after 24 h. DHA (*cis*-4,7,10,13,16,19-docosahexaenoic acid), ETA (5,8,11-eicosatriynoic acid). (E) Serum influence on system performance. Palmitic acid-inducible SEAP expression in pYL4/pYL1-transgenic HEK-293T cells cultivated in culture media containing 10% fetal calf serum (+FCS), 10% charcoal-stripped fetal bovine serum (CSFBS) or no serum (-FCS) after 24 h. All data are means ± SD (*n* = 3).

sponse element (SRE) and nuclear factor of activated T cell response element (NFAT) (29,30–32), extracellular FFA levels could be directly linked to the expression of a specific target gene (Figure 1A).

Next, we screened promoter variants with different calcium-responsive elements (31,33,34) in order to optimize the system. We constructed monomeric (P_{CRE}, P_{SRE}, P_{NFAT}, P_{NF-κB}), dimeric (P_{CRE-SRE}, P_{SRE-NFAT}, P_{AP-1-NF-κB}) and trimeric (P_{CRE-SRE-NFAT}) promoters, and observed the greatest transgene induction with P_{CRE-SRE-NFAT}. The combinatorial assembly of these three response elements might enhance the sensitivity and amplitude of calcium regulation, and therefore, maximizes the overall signal transduction mechanism of the cell (Figure 1B).

Versatility of the optimized FFA-activated transgene switch (FATS) was assessed by co-transfection of pYL4 and pYL1 into several rodent and human cells. Consistent SEAP induction with palmitic acid indicated that the system was functional in all tested cell types, including

stem cell-derived hMSC-hTERT, suggesting broad applicability of this gene control device (Figure 1C). Variations in GPR40 and related signaling protein expression (35), GPCR phosphorylation (36), cellular composition of downstream calcium signaling effectors and regulators (37,38), and protein secretion and transfection efficiencies (39) may possibly explain the different expression profiles in specific cell types and species of cell hosts. Considering the basal expression levels, maximum expression levels and induction fold, we selected two human-derived cell types, human embryonic kidney 293 cells (HEK-293T) and human bone marrow stromal cells transgenic for the catalytic subunit of human telomerase (hMSC-hTERT), for further characterization (Figure 1C).

Based on the broad sensitivity of human GPR40 to medium- and long-chain FFAs, we tested the system with a wide range of the most physiologically relevant FFAs and observed dose-dependent transcriptional activation in the concentration range from 1 to 50 μM (Figure 1D). Sig-

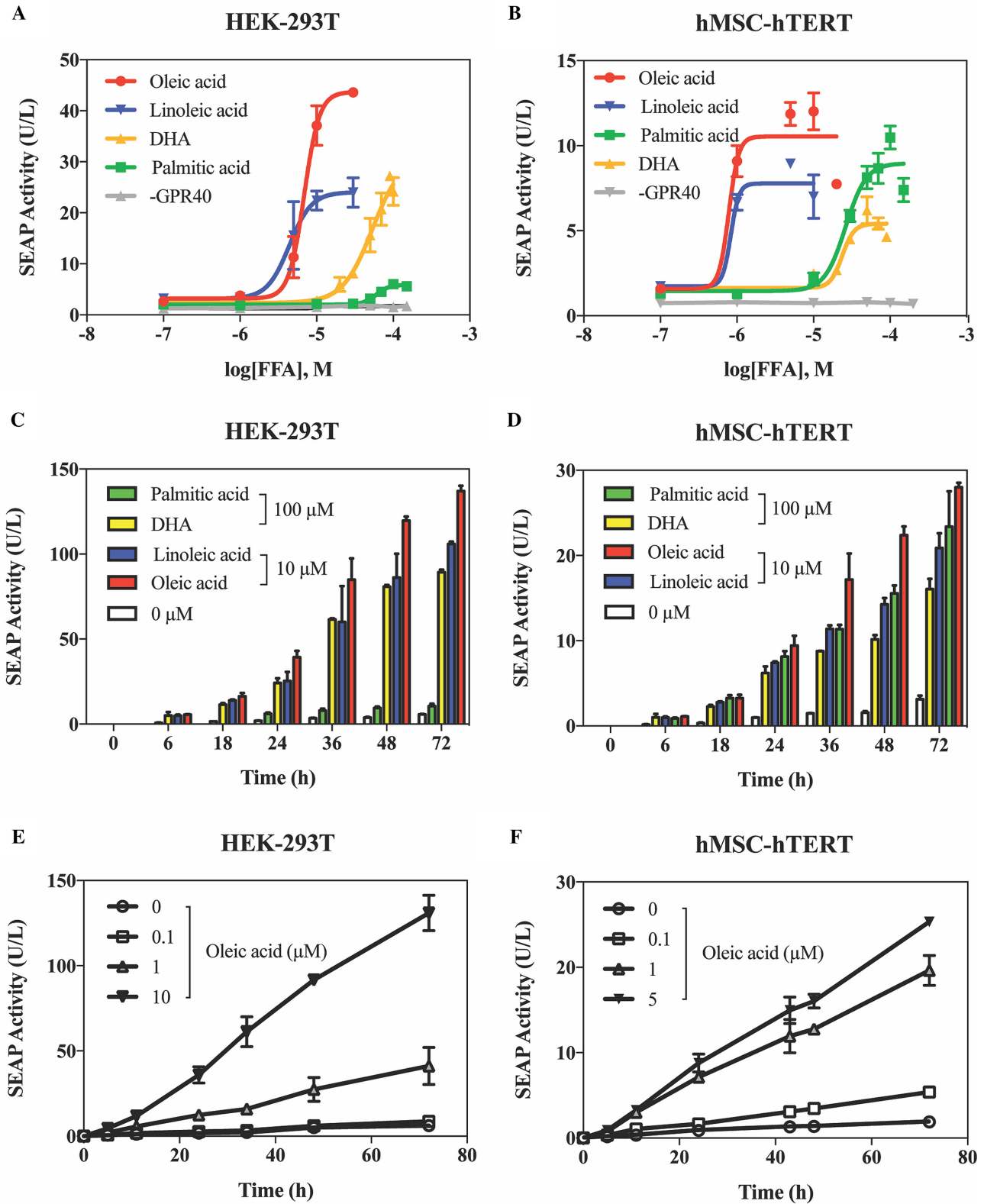


Figure 2. Responsiveness of FATS to individual fatty acids. Dose-responsive SEAP expression in pYL4/pYL1-transgenic (A) HEK-293T or (B) hMSC-hTERT cells co-cultured with specific fatty acids for 24 h. Control cells harboring only reporter (pYL1; P_{CRE-SRE-NFAT}-SEAP-pA) were stimulated with palmitic acid. Induction kinetics of FATS with individual fatty acids in (C) HEK-293T or (D) hMSC-hTERT cells transfected with pYL4/pYL1 during cultivation for 72 h. Induction kinetics of FATS with increasing amounts of oleic acid in (E) HEK-293T or (F) hMSC-hTERT cells harboring pYL4/pYL1 during cultivation for 72 h. All data are means ± SD (*n* = 3).

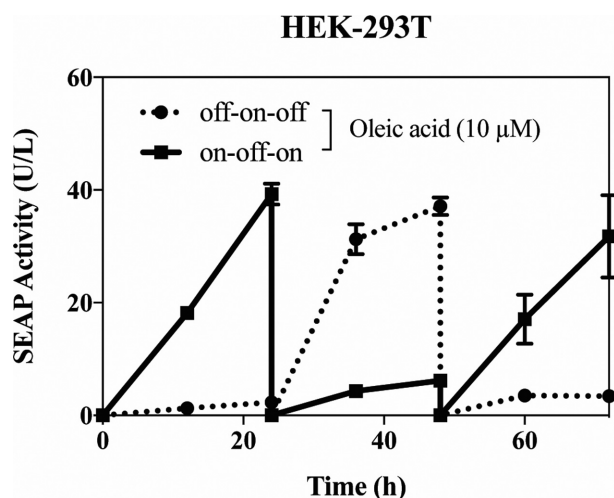


Figure 3. Reproducibility and reversibility of FATS-mediated gene expression in response to cycles of addition and removal of the inducer. pYL4/pYL1-transfected HEK-293T cells were cultured in the presence or absence of oleic acid. Every 24 h, the culture medium was exchanged and the cell density was re-adjusted. All data are means \pm SD ($n = 3$).

nificant gene induction was also observed with synthetic GPR40 agonists proposed to have clinical potential for type-2 diabetes mellitus and hepatic steatosis, i.e. GW9508 (40), TAK-875 (fasigliam) (41), and the anti-diabetic drug rosiglitazone (AvandiaTM) from the thiazolidinedione family (42) (Figure 1D). Thus, FATS may have potential applications in single drug-coordinated multiple therapeutics release, or in the combined therapy for a collective metabolic disorders.

The influence of fatty acids contained in the fetal calf serum (FCS) used to supplement standard cell culture media was assessed by cultivating pYL4/pYL1-cotransfected HEK-293T cells in medium containing no FCS or in medium containing charcoal-stripped fetal bovine serum, which is devoid of lipid-related components (Figure S1). This resulted in lower levels of basal and induced transgene expression, but had no major impact on the overall fold change of expression induced by the FATS system (Figure 1E).

Characterization of FATS *in vitro*

Detailed characterization of the FATS system was done with oleic acid (OA, C18:1), palmitic acid (PA, C16:0), linoleic acid (LA, C18:2) and docosahexaenoic acid (DHA, C22:6) because these FFAs represent the major types of fatty acids that are present in human blood (20). When HEK-293T and hMSC-hTERT cells co-transfected with pYL4/pYL1 were treated with increasing concentrations of OA, LA, PA and DHA, concentration-dependent expression of SEAP was observed (Figure 2A, B). HEK-293T and hMSC-hTERT cells both showed the greatest sensitivity to OA and LA among the four FFAs. The activation was fully GPR40-dependent, as cells expressing reporter only (pYL1) showed no SEAP induction at any tested concentrations of PA (Figure 2A, B). These concentrations of fatty acids that are able to regulate the circuit had no impact on cell

viability, since SEAP production by cells transfected with pSEAP2-control (P_{SV40}-SEAP-pA) showed little or no dependence on FFAs at the testing concentrations (Figure S2); any negative impact of high concentrations of FFA on the metabolism or viability of the cells would be expected to impair their overall cellular transgene expression capacity.

When assayed at different time points and at increasing dosages of FFAs, FATS exhibited fast induction kinetics, affording a response within 6 h (Figure 2C, D) and a dose-dependent SEAP expression profile within 72 h (Figure 2E, F). The transgene switch also showed excellent reversibility in response to cycles of exposure to 0 and 10 μ M OA at 24 h intervals (Figure 3).

In order to evaluate whether food-grade fat can also trigger the FATS system, we exposed pYL4/pYL1-transgenic HEK-293T and hMSC-hTERT cell cultures to different amounts of dietary fats, including vegetable oils, fish oil, milk, butter and cheese (Figure 4). The results indicated that the FATS system could be regulated by fatty foods *in vitro* in a dose-dependent manner (Figure 4).

FFA-regulated protein production in bioprocessing

Dose- and time-specific control of product gene expression in bioreactors requires the availability of gene switches responsive to trigger cues generally regarded as safe (GRAS) and licensed by the food and healthcare authorities. Fatty acids, as dietary components and cell metabolites, are therefore ideal trigger molecules in a biopharmaceutical manufacturing setting. We have tested oleic acid as a trigger compound for the timely induction of SEAP protein in HEK-293-derived serum-free suspension cultures, which are currently considered suitable for the production of viral particles for vaccines and gene therapy (43). In bioreactor operations, SEAP expression in pYL4/pYL1-transgenic HEK-293F cells was tightly repressed up to a specific concentration of added oleic acid, which could therefore be used to program the SEAP expression kinetics and the final titre of the gene product (Figure 5).

Functional validation of FATS *in vivo*

To validate the FATS system *in vivo*, we encapsulated pYL4/pYL1-transgenic HEK-293T cells in coherent alginate-poly-L-lysine-alginate microcapsules and implanted the microcapsules intraperitoneally into mice. The mice were given oleic acid at different doses (Figure 6A) or Swiss cheese (Figure 6B) by oral gavage twice daily. After 24 h, assay of blood samples showed dose-dependent and significant SEAP induction compared to controls. Next, to verify if the fat-sensor can be applied to detect pathological hyperlipidemia, we first tested the system on high fat (HF) diet-induced obesity (DIO) mice (DIO 42.79 ± 2.26 g versus ctrl 25.57 ± 0.67 g, $n = 7$, $P < 0.0001$). DIO mice bearing implant cells showed significant transgene expression after 24 h (Figure 6C). We then investigated the functionality of FATS in *db/db* mice lacking the leptin receptor, which develop type-2 diabetes (44) and show increased lipolysis of endogenous adipose stores, leading to increased levels of circulating FFAs (45). *db/db* mice implanted with microencapsulated cells showed significant transgene induction after 24 h (Figure 6D). Another common condition exhibiting

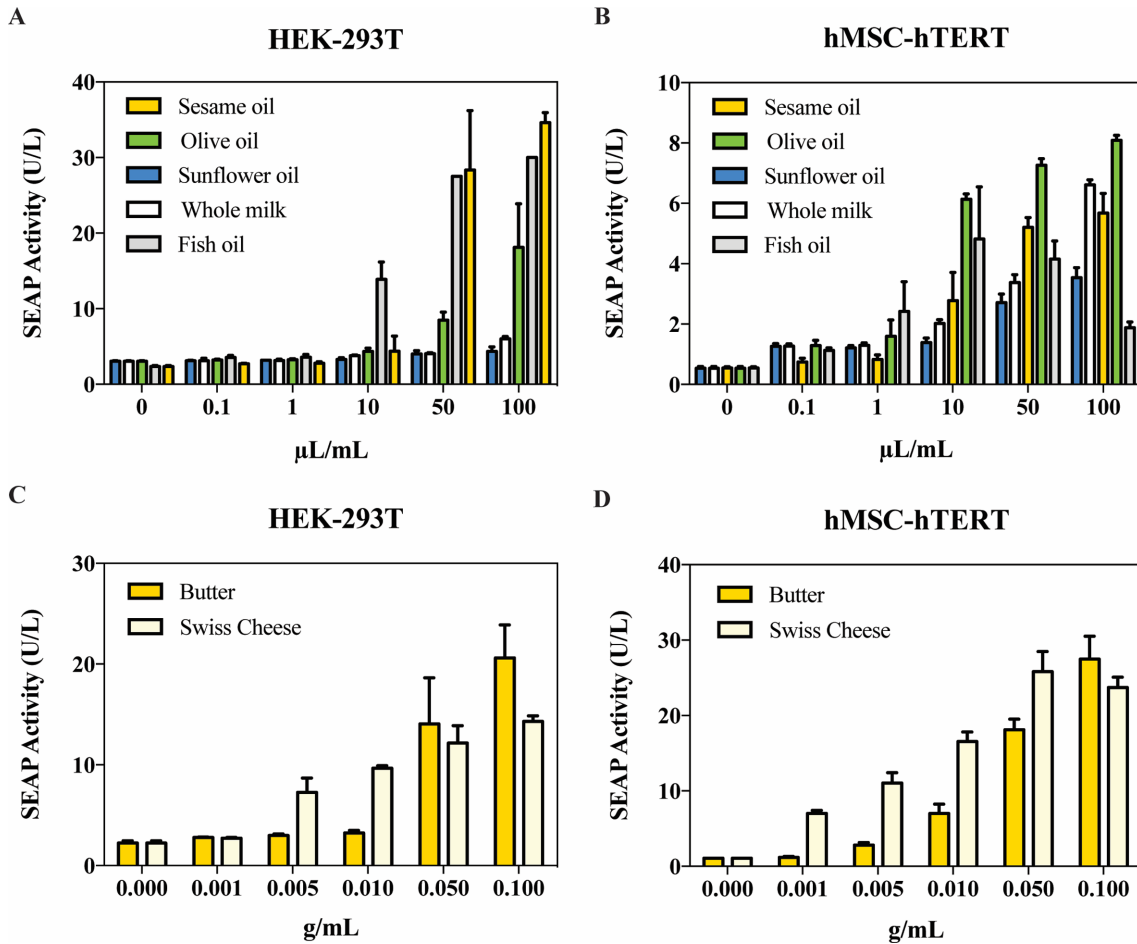


Figure 4. Dietary fat-induced SEAP expression. (A) HEK-293T and (B) hMSC-hTERT cells were transfected with pYL4/pYL1 and co-cultured with milk or oil at different concentrations for 24 h. pYL4/pYL1-transfected (C) HEK-293T and (D) hMSC-hTERT cells were cultivated in cell culture medium containing different concentrations of melted butter or cheese, and SEAP expression was assayed after 24 h. All data are means \pm SD ($n = 3$).

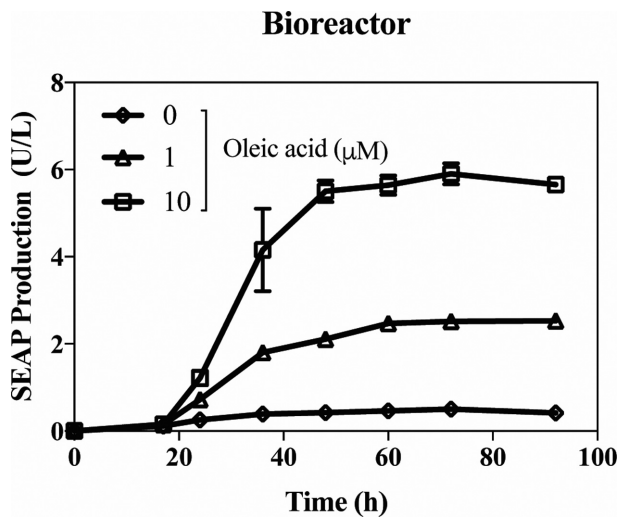


Figure 5. FFA-inducible product gene expression in bioreactors. SEAP production kinetics of pYL4/pYL1-transgenic Freestyle-293F suspension cells grown in bioreactors and triggered by the addition of 1 μ M or 10 μ M oleic acid for 92 h. Control bioreactors were run in the absence of oleic acid (0 μ M). All data are means \pm SD ($n = 3$).

high circulating fatty acids is lipodystrophy due to genetic issues, anti-retroviral medications or HIV infection (46). Long-term treatment of mice with ritonavir reproduces the clinical features of protease inhibitor-induced lipodystrophy in HIV-infected patients (47,48). Therefore, we tested the cell implants in a ritonavir-induced lipodystrophy mice model and observed significant system activation after 24 h (Figure 6E). In control mice implanted with cells constitutively expressing SEAP (pSEAP2-control), no significant difference in blood SEAP levels could be observed between non-treated animals and mice that either consumed fat or suffered from obesity (DIO), type-2 diabetes (db/db) and lipodystrophy (Figure S3). Furthermore, blood lipid levels were determined, and exhibited a good correlation with the SEAP expression profile *in vivo* (Table 2). Overall, the performance validation in mice represents a proof-of concept that the FATS system could be available as a gene switch to control desired transgene expression via intake of fatty foods. It may also be useful as a biosensor to monitor and correct pathological levels of fatty acids in a diagnostic or therapeutic setting.

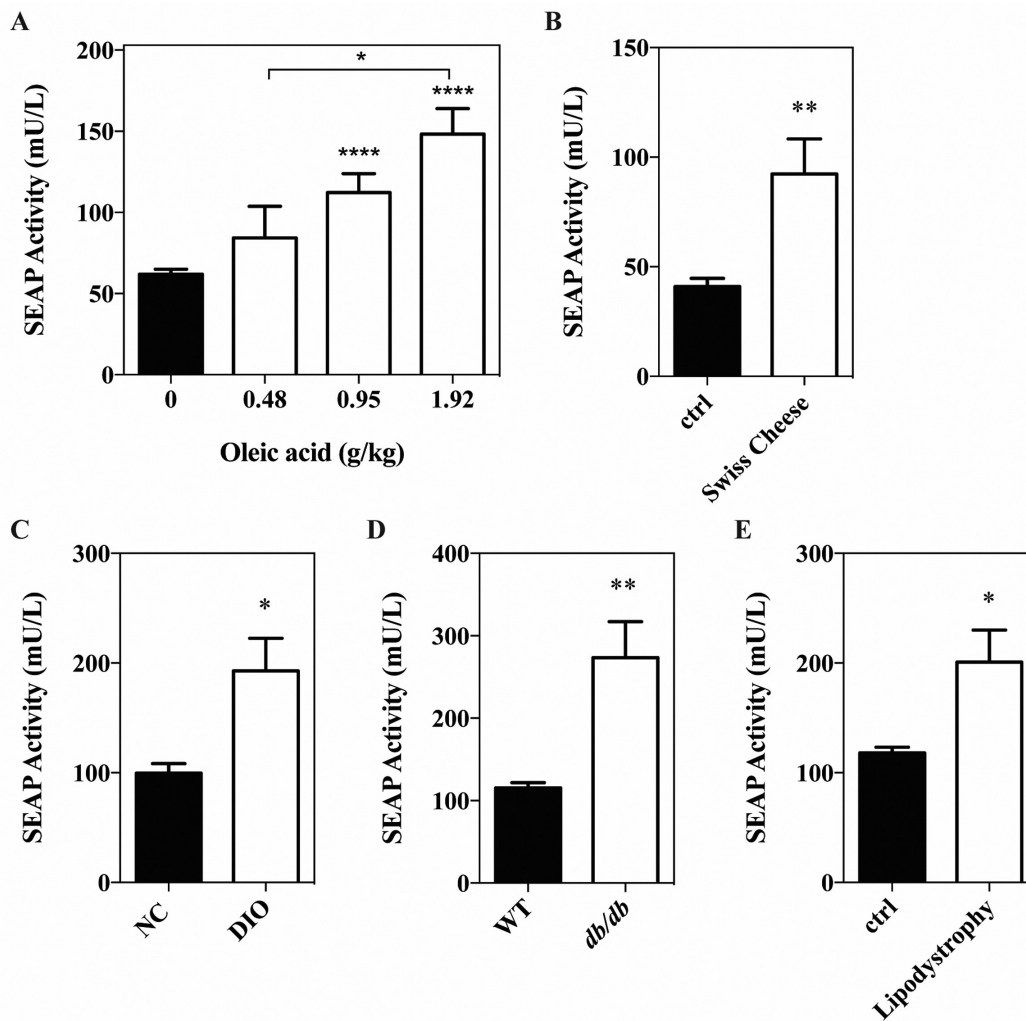


Figure 6. FATS-controlled transgene expression in mice. Mice implanted intraperitoneally with microencapsulated pYL4/pYL1-transgenic HEK-293T cells received (A) oleic acid (0 or 1.92 g/kg, $n = 14$; 0.48 or 0.95 g/kg, $n = 7$) or (B) Swiss cheese (10.7 g/kg, $n = 7$) by oral gavage twice daily, and blood SEAP activity was assayed after 24 h. (C) Mice were fed on HF diet (60 kcal% fat) (DIO) or normal chow (4 kcal% fat) (NC) for 15 weeks before receiving cell implants, and SEAP levels in the serum were assayed after 24 h ($n = 7$). (D) SEAP levels in diabetic *db/db* mice and their wild-type counterparts (WT) at 24 h after receiving cell implants ($n = 7$). (E) After receiving oral ritonavir (50 mg/kg-d) for 20 days, mice were injected with microencapsulated HEK_{pYL4/pYL1} cells and blood SEAP was assayed after 24 h ($n = 7$). Data are means \pm SEM; statistics by two-tailed *t* test; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ versus control or as indicated.

Table 2. Blood fatty acid levels quantified after 24 h in different groups of mice

Mouse group	Blood fatty acids (mmol/L)	
	-	+
oleic acid	0.548 \pm 0.031	0.681 \pm 0.037 (0.48 g/kg) ^a 0.765 \pm 0.064 (0.95 g/kg) ^b 0.945 \pm 0.065 (1.92 g/kg) ^c
Swiss cheese	0.516 \pm 0.061	0.714 \pm 0.067 ^d
DIO	0.717 \pm 0.061	1.036 \pm 0.097 ^e
<i>db/db</i>	0.816 \pm 0.045	1.373 \pm 0.146 ^f
Lipodystrophy	0.821 \pm 0.050	0.980 \pm 0.036 ^g

^a* $P = 0.017$ Mice receiving oleic acid (0.48 g/kg, $n = 7$; +) versus their vehicle control ($n = 14$; -).

^b** $P = 0.003$ Mice receiving oleic acid (0.95 g/kg, $n = 7$; +) versus their vehicle control ($n = 14$; -).

^c**** $P < 0.0001$ Mice receiving oleic acid (1.92 g/kg; +) versus their vehicle control (-) ($n = 14$).

^d* $P = 0.048$ Mice receiving Swiss cheese (+) versus their vehicle control (-) ($n = 7$).

^e* $P = 0.016$ Mice receiving high-fat diet (DIO) (+) versus mice fed on normal chow (NC) (-) ($n = 7$).

^f** $P = 0.003$ *db/db* mice (+) versus their wild-type control (-) ($n = 7$).

^g* $P = 0.025$ Lipodystrophy mice (receiving ritonavir) (+) versus their vehicle control (-) ($n = 7$).

Values are means \pm SEM for each group.

DISCUSSION

Gene switches reported so far have generally been based on prokaryotic repressors/activators together with targeted operators of the same origin (49), which raises concerns about possible pleiotropic effects in mammalian cells compared with regulatory systems derived from endogenous components (50). Indeed, adverse effects of increased immune responses to prokaryotes-derived transgene products (e.g. the widely used tetracycline-dependent systems) have been reported in many non-human primates, such as complete loss of transgene regulation and expression (51,52) and the development of anaphylactic reactions in humans, including cardiac arrest (53). Moreover, the triggering compounds of conventional gene switches, such as antibiotics (28,54), immunosuppressive agents (55), drugs (56) or hormones and their derivatives (57,58), may have secondary therapeutic effects and collateral side effects (59) including physiological disruption of the host and development of antibiotic resistance (60). There are also economic considerations associated with the elimination of inducer molecules during downstream purification of biopharmaceuticals (61). All of these issues have greatly hindered the widespread implementation of gene switches in biopharmaceutical manufacturing and biomedical applications (62). On the other hand, by targeting on endogenous metabolites as regulatory compounds and employing exclusively human-derived cellular components as building blocks, FATS can directly communicate with patients' metabolic networks to interface with and respond to endogenous signals, and so offers better biocompatibility than previous hybrid systems (63). The transmembrane localization and broad sensing capacity of GPR40 also equip the system with higher sensitivity (down to 0.1 μ M) than previously reported nuclear-receptor based system (63) (Figure S4).

The functionality of the FATS device using a safe trigger molecule to achieve timely remote control of product gene expression in a proof-of-concept bioprocessing manufacturing setting suggests that FATS has great potential for application in manufacturing drug and tool proteins (64) without introducing major issues regarding downstream purification, validation, or approval.

Furthermore, the versatile adaptability of FATS to different mouse models indicates that it is a promising candidate for future diagnostic and therapeutic applications. Free fatty acid levels in the blood are normally regulated to within a certain range in healthy individuals. However, a pathological increase can be maintained for a long time without causing any significant signs or symptoms, while current clinical discrete measurements are far from ideal either for early diagnosis or for guiding preventive measures (65,66).

Specifically, dietary interventions through food monitoring are often unsuccessful due to the hidden nature of many fats, the variation of type and content of fatty acids contained in foods, and the sensitivity of individuals to questions about fat intake in their diets (67). For these reasons, a fat-sensor to reflect endogenous fatty acid levels through a readily accessible reporter molecule would be particularly desirable in real clinical settings. This is impor-

tant, because elevated blood concentrations of fatty acids increase oxidant stress, promote endothelial dysfunction, induce inflammatory cytokine release and provoke insulin resistance (68). High blood FFA levels are seen in obesity (69), insulin resistance (70), type 2 diabetes mellitus (71), cardiovascular disease (72) and hepatic steatosis (73), and moreover, interventions to decrease circulating fatty acid levels have shown great therapeutic value in improving insulin sensitivity, normalizing glucose homeostasis in type-2 diabetes mellitus and correcting dyslipidemia in cardiovascular complications (74). When linked to the production of a hypolipidemic agent (75–77), the fat-sensor could be customized to enable versatile therapeutic interventions and self-autonomously restore blood lipid homeostasis in populations highly susceptible to hyperlipidaemia and its complications. For example, the present findings in lipodystrophic mice suggest the feasibility of an alternative FATS-controlled cell-based therapy for lipotrophic diabetes via combined expression of leptin and adiponectin, which has been shown to completely reverse insulin resistance in lipotrophic mice (78). Furthermore, by changing the output protein to ghrelin (79) or progestins (80), the circuit might be applied to prevent cancer cachexia patients from experiencing significant whole-body lipolysis in the late stage of chemotherapy (81). Thus, we believe the FATS system, combining the use of a physiologically compatible inducer, precise and reversible transcription tunability, and broad functionality in different cell types or in bioreactors, as well as in animals, has a wide range of potential applications in advanced protein manufacturing, diagnosis of pathological blood-fat levels as well as gene- and cell-based therapies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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