Cosmetics-triggered percutaneous remote control of transgene expression in mice

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ABSTRACT

Synthetic biology has significantly advanced the rational design of trigger-inducible gene switches that program cellular behavior in a reliable and predictable manner. Capitalizing on genetic components, including the repressor PmeR and its cognate operator OPmeR, that has evolved in \textit{Pseudomonas syringae pathovar tomato} DC3000 to sense and resist plant-defence metabolites of the paraben class, we have designed a set of inducible and repressible mammalian transcription-control devices that could dose-dependently fine-tune transgene expression in mammalian cells and mice in response to paraben derivatives. With an over 60-years track record as licensed preservatives in the cosmetics industry, paraben derivatives have become a commonplace ingredient of most skin-care products including shower gels, cleansing toners and hand creams. As parabens can rapidly reach the bloodstream of mice following topical application, we used this feature to percutaneously program transgene expression of subcutaneous designer cell implants using off-the-shelf commercial paraben-containing skin-care cosmetics. The combination of non-invasive, transdermal and orthogonal trigger-inducible remote control of transgene expression may provide novel opportunities for dynamic interventions in future gene and cell-based therapies.

INTRODUCTION

Synthetic trigger-controlled gene switches that enable spatio-temporal fine-tuning of transgene expression have been instrumental for functional genomic research (1), drug discovery (2) and the manufacturing of difficult-to-produce drug targets (3) and protein therapeutics (4). During the past decade synthetic biology, the science of re-assembling cataloged and standardized biological items in a systematic, rational and predictable manner to create, engineer and program functional biological designer devices, systems and organisms with novel and useful functions (5–10) has significantly advanced the design of gene switches. They evolved from simple control devices providing trigger-inducible transgene expression (11–15) to complex transcription/translation networks enabling oscillating expression dynamics (16), intercellular communication (17) and fundamental arithmetic operations (18,19). Today, gene switches form the basis for the design of therapeutic gene networks that have been successfully validated in cell-based therapies using animal models of prominent human disorders (2,4,20–29).

Short-chain alkylated parabens are a group of plant antimicrobial defense metabolites (e.g. methylparaben (MP) is found in oca and grapefruit seeds, (30,31)) that have been clinically licensed by the FDA as well as approved within the European Union as food additives (E218, MP; E214, ethylparaben (EP); E216, propylparaben (PP); E209, heptylparaben) and have been widely used for over 60 years as preservatives in food, cosmetics and pharmaceuticals (32,33). Parabens (i) are inexpensive due to their simple high-volume industrial production, (ii) transdermally absorbed (34–36), (iii) rapidly reach the bloodstream (33,36), (iv) are rapidly metabolized and (v) renally cleared and (vi) are generally regarded as safe (37).

We have engineered paraben-repressible and -inducible transgene expression systems based on the genetic componentry of the Gram-negative bacterium \textit{Pseudomonas syringae pathovar tomato} DC3000, a plant pathogen that causes bacterial specks of tomato (38). Expression of \textit{P. syringae}'s major multidrug efflux pump MexAB-OprM is regulated by PmeR (\textit{Pseudomonas} multidrug efflux regulator), a TetR-type transcriptional repressor that binds to an inverted repeat (OPmeR) overlapping with the promoters driving \textit{mexAB-oprM} and \textit{pmeR} (39,40). Parabens...
have been shown to induce the expression of the mexAB-oprM genes by binding to PmeR and disrupting the PmeR–OprM interaction, thereby conferring resistance to multiple plant defense metabolites including parabens (40,41). Taking advantage of the paraben-responsive PmeR–OPmeR interaction, we have designed a set of mammalian gene switches that allow paraben-repressible as well as inducible transgene expression in a variety of human cell lines. Furthermore, topical application of commercial paraben-containing skincare products was able to remote control transgene expression in subcutaneous (SC) designer cell implants in mice, suggesting that this technology will be compatible with future clinical applications.

**MATERIALS AND METHODS**

**Plasmid design**

Comprehensive design and construction details for all expression vectors are provided in Table 1. The assembly of some plasmids required annealing of complementary oligonucleotides: 50 pmol of each oligonucleotide was mixed in 50 µl H2O-diluted 1x NEB Buffer 4 (New England Biolabs, Ipswich, MA, USA), heated for 10 min at 95°C, cooled down over 4 h to 22°C and incubated at 22°C for another 2 h prior to cloning into the corresponding vector backbone. All relevant genetic components have been confirmed by sequencing (Microsynth, Balgach, Switzerland).

**Cell culture and transfection**

Human embryonic kidney cells (HEK-293T, ATCC: CRL-11268), human cervical adenocarcinoma cells (HeLa, ATCC: CCL-2), human fibrosarcoma cells (HT-1080, ATCC: CCL-121), telomerase-immortalised human mesenchymal stem cells (hMSC-TERT, (42)) and baby hamster kidney cells (BHK-21, ATCC: CCL-10) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Basel, Switzerland; cat. no. 52100–39) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution. All cell lines were cultured in a humidified atmosphere containing 5% CO2.

**Construction and characterization of stable cell lines**

The HEK-PAROFF cell line, transgenic for paraben-repressible secreted alkaline phosphatase (SEAP) expression, was constructed by co-transfection of HEK-293 cells with a 10:5:1 (w/w/w) mixture of pWH8 (PSV40-PMA-pA), pWH10 (PMPA-SEAP-pA) and pZeoSV2(+) (PSV40-zeo-pA), followed by selection in culture medium containing 1 mg/ml zeocin (Invitrogen, cat. no. R250-05) and FACS-mediated single-cell cloning. Six out of 30 cell clones were randomly picked and the best-in-class HEK-PAROFF was used for all follow-up studies. Likewise, the HEK-PARON cell line, transgenic for paraben-inducible SEAP expression, was constructed by co-transfection of HEK-293 cells with an 8:8:1 (w/w/w) mixture of pWH9 (PSV40-PMSP-pA), pWH5 (PMS-SEAP-pA) and pZeoSV2(+) (PSV40-zeo-pA) followed by selection in culture medium containing 1 mg/ml zeocin and FACS-mediated single-cell cloning. Six out of 30 cell clones were randomly chosen and the best-in-class HEK-PARON cell line was used for all follow-up studies.

**Quantification of reporter protein production**

Production of human placental SEAP was quantified in the culture supernatant by measuring the colorimetric absorbance time course of the SEAP-mediated p-nitrophenylphosphate to p-nitrophenolate conversion, as described previously (44). In brief, 120 µl of buffered substrate solution (100 µl of 2x SEAP assay buffer [20 mM homoarginine, 1 mM MgCl2, 21% diethanolamine, pH9.8] and 20 µl substrate solution [120 mM p-nitrophenylphosphate]) was added to 80 µl heat-inactivated (65°C, 30 min) cell culture supernatant and the light absorbance time course was measured at 405 nm (37°C). The SEAP levels in the bloodstream were profiled using a chemiluminescence-based assay (Roche Diagnostics GmbH, Mannheim, Germany; cat. no. 11 779 842 001).

**Chemicals and cosmetics**

Ethanol (EtOH; cat. no. 02860), dimethyl sulfoxide (DMSO; cat. no. D8418), MP (cat. no. H3647), EP (cat. no. 11988), PP (cat. no. P53357), butylparaben (BP; cat. no. 54680), isobutylparaben (iBP; cat. no. 715077) and phloretin (cat. no. P7912) were purchased from Sigma-Aldrich (Buchs, Switzerland). All parabens were prepared and stored as 20 mM stock solutions in 50% EtOH (in ddH2O). Phloretin was prepared and stored as a 50 mM stock solution in 100% EtOH. For animal experiments, different doses of PP were prepared in 100% DMSO before the treatment. Kamill® hand cream (Kamill® Hand & Nagelcreme Classic; Burnus, Darmstadt, Germany) was diluted 200x in 25% DMSO (in ddH2O). Cien® shower gel (Cien® spring bloom magic shower gel; Lidl Stiftung & Co. KG, Neckarsulm, Germany) and Dove® shower gel (Dove® Dusche Schutzende Pflege; Unilever, Hamburg, Germany) were diluted 100x in 25% DMSO (in ddH2O). Lancaster® cleanser (Lancaster® Express Cleanser, Lancaster, Paris, France) and Lancôme Paris® toner (Lancôme Paris® soft-
Table 1. Plasmids and oligonucleotides designed and used in this study

| Plasmid                  | Description                                                                 | Reference |
|-------------------------|-----------------------------------------------------------------------------|-----------|
| pSEAP2-Control          | Constitutive mammalian SEAP expression vector (P SV40-SEAP-pA)              | Clontech, CA |
| pUC57                   | pUC19-derived prokaryotic expression vector                                | GeneScript, NJ |
| pZeoSV2(+)              | Constitutive mammalian SEAP expression vector encoding the zeocin resistance gene (P pmcVmy-zeo-pA). | Invitrogen, CA |
| pMG11                   | Constitutive mammalian TgA expression vector (P SV40-TgA1-pA).             | (12)      |
| pSAM200                 | Constitutive mammalian TTA expression vector (P SV40-TTA-pA).              | (14)      |
| pWH1/24                 | γ-butyrolactone (SCBI)-repressible SEAP expression vector (P SCBI-SEAAP-pA). | (15)      |
| pKR71                   | Constitutive mammalian KstR-KRAB expression vector (P SV40-KstR-KRAB-pA). | unpublished |
| pMM15                   | Mammalian expression vector (P SV40-pA).                                   | unpublished |
| pMX101                  | Constitutive mammalian TgS expression vector (P SV40-TgS-pA; TgS, TgR-KRAB). | This work |
|                         |                                                                             |           |
| Plasmids and oligonucleotides: Restriction endonuclease-specific sites are underlined, annealing base pairs are indicated in capital letters and the operator module Opar III is shown in bold. Abbreviations: KRAB, Krueppel-associated box protein of the human kox-1 gene; KstR, Mycobacterium tuberculosis repressor of the cholesterol biosynthesis; Opar III, SCA-specific operator; Opar II, tandem TgR-specific operator; pA, polyadenylation site; PSCBI, human cytochrome c oxidase immediate early promoter; P pmcVmy, minimal version of P pmcV; PAPA, PmR-derived paraben-dependent transactivator (PAPA-PRM); PAPA-SCBI, PmR-derived paraben-dependent transactivator (PAPA-PRM); PAPA, γ-butyrolactone (SCBI)-repressible promoter (P APA-pA; APA, paraben-repressible promoter (O apa-pA); PAPA, paraben-inducible promoter (P APA-pA); PAPA, simian virus 40 promoter; PAPA, phloretin-inducible promoter; PAPA-SCBI-APA, SCBI-dependent transactivator (SCBI-APA); PAPA, Streptomyces coelicolor-1,1-(hydroxy-6-methylenephtyl)-3-hydroxyphthalate; PAPA, Streptomyces coelicolor γ-butyrolactone (SCBI)-specific quorum-sensing receptor; SEAP, human placental secreted alkaline phosphatase; TetR, Escherichia coli Tetr-tetR-dependent tetracycline-dependent repressor of the tetracycline resistance gene; ITA, tetracycline-dependent transactivator (TetR-VP16); TgS, TgR, repressor of the Pseudomonas putida DOT-T1E ABC multidrug efflux pump; TgS, TgR, TgR-dependent phloretin-dependent transsilencer (TgR-KRAB); VP16, Herpes simplex virus-derived transactivation domain; zeo, zeocin resistance gene. |

Animal experiments

Designer cell implants were produced by microencapsulating pWH9/pWH5-transgenic HEK-293 into coherent alginate-poly-(L-lysine)-alginate beads (400 μm diameter; 200 cells/capsule) using an Inotech Encapsulator Research Unit IE 50R (Buechi Labortechnik AG, Flawil, Switzerland) set to the following parameters: 0.2 mm nozzle with a vibration frequency of 1025 Hz, 25-ml syringe operated at a flow rate of 410 units and 1.12 kV for bead dispersion (26). One hour after intraperitoneal (IP) or subcutaneous (SC) injection of the alginate microparticles into eight-week-old female OF1 mice (incnis France souche 1; Charles River Laboratories, France), the animals were treated with IP injections (0–10 mg/kg in 50 μl DMSO, once every 24 h) or topical application of commercial hand cream (Kamil®, 600 mg, 3x every 8 h) and cleanser (Lancaster®, 600 μl, 3x every 8 h) or solutions containing three different concentrations of PP (50 μl, 3x every 8 h for a total of 0, 10, 100 mg/kg day−1). Blood samples were collected 48 h after implantation and serum was isolated using microtainer serum separating tubes (SST) tubes according to the manufacturer's instructions (centrifugation for 5 min at 10 000g; Becton Dickinson, Plymouth, UK; cat. no. 365967). Semi-quantitative analysis of blood-paraben levels was performed by injecting mice once with 300 mg/kg of PP, collecting blood samples after 24 h and adding 10 μl of serum to 2.5x 10^8 pWH9/pWH5-transfected HEK-293 cells, before SEAP expression was profiled and compared to a standard curve after 48 h. All experiments involving animals were performed according to the directive of the European Community Council (2010/63/EU), approved by the French Republic (no.
Regulation of PAR\textsubscript{OFF}:

\begin{align*}
\text{(A)} \quad \text{pWH8} & \quad \begin{array}{c}
\text{P}_{\text{SEAP}} \rightarrow \text{PmeR} \rightarrow \text{VP16} \rightarrow \text{pA} \cdots \text{PMA}
\end{array} \\
\text{pWH10} & \quad \begin{array}{c}
\text{OP}\text{meR}_2 \rightarrow \text{P}_{\text{PhCMVmin}} \rightarrow \text{SEAP} \rightarrow \text{pA} \\
\text{P}_{\text{PMA}}
\end{array}
\end{align*}

\begin{align*}
\text{Regulation of PAR\textsubscript{ON}:
}
\begin{align*}
\text{(B)} \quad \text{pWH9} & \quad \begin{array}{c}
\text{P}_{\text{SEAP}} \rightarrow \text{PmeR} \rightarrow \text{KRAB} \rightarrow \text{pA} \cdots \text{PMS}
\end{array} \\
\text{pWH5} & \quad \begin{array}{c}
\text{OP}\text{meR}_2 \rightarrow \text{SEAP} \rightarrow \text{pA} \\
\text{P}_{\text{PMS}}
\end{array}
\end{align*}
\end{align*}

\begin{align*}
\text{- Parabens} & \quad \begin{array}{c}
\text{OP}\text{meR}_2 \rightarrow \text{P}_{\text{PhCMVmin}} \rightarrow \text{SEAP} \rightarrow \text{pA} \\
\rightarrow \text{ON}
\end{array} \\
\text{+ Parabens} & \quad \begin{array}{c}
\text{OP}\text{meR}_2 \rightarrow \text{P}_{\text{PhCMVmin}} \rightarrow \text{SEAP} \rightarrow \text{pA} \\
\rightarrow \text{OFF}
\end{array}
\end{align*}

\begin{align*}
\text{(C)} \quad \text{SEAP Production (U/L)} & \quad \begin{array}{c}
\begin{array}{c}
\text{0} \% \text{pWH8/pWH9} \\
25 \% \text{pWH8} \\
50 \% \text{pWH8} \\
25 \% \text{pWH9} \\
50 \% \text{pWH9}
\end{array}
\end{array} \\
\text{Time (h)} & \quad \begin{array}{c}
12 \quad 24 \quad 36 \quad 48 \quad 60
\end{array}
\end{align*}

\begin{align*}
\text{(D)} \quad \text{Viable Cell Number (x10^7/mL)} & \quad \begin{array}{c}
\begin{array}{c}
\text{0} \% \text{pWH8/pWH9} \\
25 \% \text{pWH8} \\
50 \% \text{pWH8} \\
25 \% \text{pWH9} \\
50 \% \text{pWH9}
\end{array}
\end{array} \\
\text{Time (h)} & \quad \begin{array}{c}
12 \quad 24 \quad 36 \quad 48 \quad 60
\end{array}
\end{align*}
Figure 1. Design and validation of paraben-controlled mammalian transgene expression systems. (A) Design and functionality of the paraben-repressible transcription-control system PAROFF. The synthetic mammalian paraben-mediated transactivator PMA (pWH8, PSV40-PMA-pA; PMA, PmeR-VP16) was designed by C-terminal fusion of PmeR (Pseudomonas multidrug efflux regulator) to the Herpes simplex virus-derived transactivation domain VP16. Following constitutive expression by pSV40, PMA binds and activates the chimeric promoter PPM (pWH10, PPM-SEAP-pA; PPM, OPmeR2-PhCMVmin) containing a tandem PMA-specific operator module OPmeR2 5' of PhCMVmin, which is set to drive expression of SEAP. In the absence of parabens PMA binds PPM and drives SEAP expression, while the presence of paraben derivatives results in the release of PMA from OPmeR2 and represses SEAP expression.

(B) Design and functionality of a paraben-inducible transcription-control system PARON. The synthetic mammalian paraben-mediated transsilencer PMS (pWH9, PSV40-PMS-pA; PMS, PmeR-KRAB) was designed by C-terminal fusion of PmeR to the transsilencing Krueppel-associated box (KRAB) domain of the human kox-I gene. Following constitutive expression by pSV40, PMS binds and silences a chimeric promoter PPM (pWH5, PPM-SEAP-pA; PPM, PSV40-OPmeR2) containing a tandem PMS-specific operator module OPmeR2 3' of a constitutive PSV40. In the absence of parabens PMS binds and silences PPM-driven SEAP expression, while the presence of paraben derivatives results in the release of PMS from PPM and induces PPM-driven SEAP expression.

(C) Reporter protein-based metabolic integrity assay and (D) cell proliferation assay of PMA- and PMS-expressing cells. HEK-293 cells were co-transfected with pSEAP2-Control (0.3 μg, w/w), different amounts of pWH8 (PSV40-PMA-pA) or pWH9 (PSV40-PMS-pA) (0–0.3 μg, w/w) and optionally with the isogenic empty vector pMM15 that serves as a filler plasmid to keep the total transfected DNA constant (0.6 μg). The resulting SEAP levels (C) and viable cell numbers (D) of the transfected HEK-293 populations were profiled for up to 60 h.

(E) Viability of human cells after exposure to different paraben derivatives. HEK-293 cells were cultivated in medium containing 0–200 μM of different paraben derivatives (MP, methylparaben; EP, ethylparaben; PP, propylparaben; BP, butylparaben; iBP, isobutylparaben) for 48 h before cell viability was scored.

(F) Reporter protein-based metabolic integrity assay for paraben derivatives. HEK-293 cells were transfected with the constitutive SEAP expression vector pSEAP2-Control and cultivated in medium containing 0–200 μM of different parabens (MP, methylparaben; EP, ethylparaben; PP, propylparaben; BP, butylparaben; iBP, isobutylparaben) for 48 h before SEAP levels were profiled in the culture supernatant.

(G) Dose-dependent paraben-repressible SEAP expression (PAROFF system). HEK-293 cells were co-transfected with pWH8 and pWH10 and cultivated for 48 h in medium containing 0–200 μM of different parabens (MP, methylparaben; EP, ethylparaben; PP, propylparaben) in the absence of parabens PMA binds PPM and drives SEAP expression, while the presence of paraben derivatives results in the release of PMA from OPmeR2 and represses SEAP expression.

(H) Dose-dependent paraben-inducible SEAP expression (PARON system). HEK-293 cells were co-transfected with pWH8 and pWH5 and cultivated for 48 h in medium containing 0–200 μM of different parabens (MP, methylparaben; EP, ethylparaben; PP, propylparaben) before SEAP levels were profiled in the culture supernatant. Repression factors: MP: 48; EP: 106; PP: 175. All data are shown as the mean ± SD, n = 3 independent experiments.
RESULTS

Design, construction and validation of paraben-repressible and -inducible mammalian transgene expression switches

In Pseudomonas syringae pv. tomato DC3000, the repressor PmeR (Pseudomonas multidrug efflux regulator) is released from promoters containing specific OPmeR operator sites upon interaction with plant defense metabolites of the paraben class, to induce the paraben-eliminating multidrug efflux pump MexAB-OprM and establish resistance to plant-derived antimicrobial compounds (39,40). Capitalizing on the paraben-sensitive PmeR–OpmeR interactions, we have designed two isogenic synthetic mammalian gene switches that either repress (PAROFF) or induce (PARON) transgene expression in response to FDA-approved parabens.

PAROFF consists of the synthetic mammalian transcription factor PMA (paraben-mediated transactivator; pWH8, Psv40-PMA-pA; PMA, Pmer-VP16), engineered by fusing PmeR’s C-terminus to the Herpes simplex virus-derived transactivation domain (VP16), that modulates the activity of synthetic PPM promoters (pWH10, PPM-SEAP-pA; PPM, OPmeR2-P(CMV)in), containing the PMA-specific tandem operator module OPmeR2 5’ of a minimal version of the human cytomegalovirus immediate-early promoter (P(CMV)in), in a paraben-responsive manner: In the absence of parabens, PMA binds and activates PPM-driven transgene expression while paraben derivatives prevent the PMA–PPM interaction and repress target-gene expression (Figure 1A).

PARON consists of a synthetic mammalian transcription silencer PMS (paraben-mediated transsilencer; pWH9, Psv40-PMS-pA; PMS, PmeR-KRAB), engineered by fusing PmeR’s C-terminus to the Kruppep-associated box (KRAB) domain of the human kox-1 gene, that modulates the activity of synthetic PPSM promoters (pWH5, PPSM-SEAP-pA; PPSM, Psv40-OPmeR2), containing the PMS-specific tandem operator module OPmeR2 3’ of the constitutive simian virus 40 promoter (Psv40), in a paraben-responsive manner. In the absence of parabens PMS binds and activates PPSM-driven transgene expression while paraben derivatives prevent the PMS–PPSM interaction and induce target-gene expression (Figure 1B).

To assess the impact of PMA and PMS expression on the metabolic integrity and the viability of mammalian cells, we co-transfected HEK-293 with pSEAP2-Control and increasing concentrations of either the PMA-encoding vector pWH8 (Psv40-PMA-pA) or the PMS-encoding vector pWH9 (Psv40-PMA-pA) and profiled SEAP production (Figure 1C) as well as the viable cell number of the cell population for up to 60 h (Figure 1D). Likewise, to assess the impact of parabens on the viability and the metabolic integrity of mammalian cells, we cultivated HEK-293 cells in medium containing different concentrations (0–200 μM) of MP, EP, PP, BP and iBP, which are most commonly used as cosmetics additives, and profiled the percent viable cell number (Figure 1E) and the overall SEAP production capacity of treated cell lines (Figure 1F). Collectively, these results show that none of the parabens impaired cell viability and that only parabens with longer alkyl chains such as BP and iBP decreased constitutive SEAP expression, suggesting that they have a negative impact on the cell physiology within the tested concentration range (0–200 μM). However, neither MP, EP nor PP did impair the SEAP production capacity of HEK-293 (Figure 1F). When using increasing concentrations (0–200 μM) of MP, EP and PP to control SEAP expression in PAROFF (pWH8/pWH10) or PARON (pWH9/pWH5)-engineered HEK-293 cells product gene expression was repressed (Figure 1G) or induced (Figure 1H) in a dose-dependent manner, respectively. Since PP showed the tightest repression and highest induction for PAROFF as well as PARON, this paraben derivative was used in all follow-up experiments.

Validation of PAROFF- and PARON-controlled transgene expression in different mammalian cell lines

To assess the potential of the paraben-responsive transgene expression devices for a wide range of applications, we tested the performance of the PAROFF and PARON systems in different cell types. Therefore, the PAROFF system (pWH8/pWH10) was transfected into four human (HEK-293, HeLa, HT-1080, hMSC-TERT) and two animal (CHO-K1, BHK-21) cell lines and cultivated for 48 h in the presence and absence of 200 μM PP before SEAP was profiled in the culture supernatants. The PAROFF system was functional in all tested cell lines and showed the best regulation performance in HeLa and HEK-293 cells, combining highest absolute SEAP expression levels (HeLa: 71.1 ± 4.4 U/L; HEK-293: 99.6 ± 3.8 U/L) in the PP-free induced state with almost complete PP-mediated repression (HeLa: 0.2 ± 0.3 U/L; HEK-293: 4.3 ± 0.1 U/L) (Figure 2A).

Likewise, when cultivating the same set of mammalian cell lines engineered with the PARON system (pWH9/pWH5) for 48 h in the presence and absence of 200 μM PP, resulting SEAP expression levels in the supernatant indicated that the PARON system too was functional in a wide variety of cell types. However, the PARON gene switch showed the optimal PP-inducible expression control in HEK-293 cells with minimal SEAP expression (1.2 ± 1.0 U/L) in the absence and high maximum expression induction (210.2 ± 10.9 U/L) in the presence of the trigger compound (Figure 2B).

Collectively, both ON-type and OFF-type PP-controlled transgene expression systems are functional in different mammalian cell types from various species, suggesting that these control devices will have broad utility for a wide range of applications.

Construction, selection and characterization of stably transgenic paraben-regulated mammalian cell lines

We have generated six clonal double-transgenic cell lines (HEK-PAROFF1-6), in which SEAP is controlled by the PAROFF system, by stably co-transfecting pWH8 and pWH10 into HEK-293. All of these transgenic HEK-PAROFF cell lines showed PP-repressible regulation pro-
files, but differed in their overall SEAP expression performance characterized by specific maximum and basal transgene expression signatures (Figure 3A). Due to the combination of the highest induction ratio and the lowest IC50 concentration of PP (Figure 3B), HEK-PAROFF1 emerged as the best-in-class transgenic cell line that was chosen for all follow-up studies. Also, HEK-PAROFF1 showed dose-dependent SEAP repression (Figure 3B), dose-dependent SEAP induction kinetics (Figure 3C) and completely reversible SEAP expression profiles when alternating the presence and absence of PP in the culture medium (Figure 3D).

Likewise, we have generated six clonal double-transgenic cell lines, in which SEAP is controlled by the PARON system, by stably co-transfecting pWH9 and pWH5 into HEK-293. All of these transgenic HEK-PARON cell lines showed PP-inducible regulation profiles, but differed in their overall SEAP induction ratio (Figure 3E). The cell clone HEK-PARON1 showed (i) a near perfect induction ratio characterized by almost undetectable basal expression in the absence of PP and high maximum expression levels in the presence of PP (Figure 3F), (ii) robust PP dose-dependent SEAP production kinetics (Figure 3G) and completely reversible SEAP expression kinetics when alternating presence and absence of the trigger compound in the culture medium (Figure 3H).

Because of the integration of the transgene expression units into random chromosomal loci by illegitimate recombination, gene switch performance is dependent on the chromosomal context and can therefore dramatically vary among different stable cell clones (Figure 3A and E) (45,46).

Comparative performance analysis of the PARON and PEACEON systems

ON-type gene control systems that induce target-gene expression in response to a transient molecular cue is the preferred gene switch design, because the trigger compound only needs to be administered upon induction. In contrast, OFF-type switches require continuous presence of control compounds for repression and active removal for induction, which limits applications of this control topology in vivo. However, ON-type switches are more challenging to design, as they have to be extremely tight so that the target protein does not accumulate to significant levels even in the absence of the trigger compound. We have therefore redesigned the phloretin-adjustable control element (PEACE), the pioneering OFF-type design that enabled transdermal control by the apple metabolite phloretin (12), into an isogenic ON-type design (pMX101, PSV40-TtgR-KRAB-pA; pWH9, PSV40-OTtgRSEAP-pA) for comparative performance analysis with the PARON system (pWH9/pWH5) (Figure 4). To assess the impact of phloretin on the viability and the metabolic integrity of mammalian cells, we performed the same reporter protein-based assay as for parabens (Figure 1E and F) and found that although phloretin did not impair the viable fraction of treated cells within the standard PEACE-inducing concentration range (0–50 µM, (12)) (Figure 4A), higher concentrations decreased constitutive SEAP production capacity of mammalian cells (Figure 4B). Therefore, the phloretin-inducible transgene expression switch (Figure 4C) may not reach optimal peak expression levels in various human cell lines. Also, the induction factor reached by the PEACEON system was lower in all tested mammalian cell lines (Figure 4D) when compared to the PARON system (Figure 2B). Moreover, the PP-controlled transgene expression system delivers precisely adjustable induction kinetics (Figure 4E) and shows improved dose-dependent transgene-induction characteristics compared to its phloretin-regulated counterpart (Figure 4F). Collectively, these data suggest that the PARON gene expression system will be the preferred control design for percutaneous control of transgene expression.

Figure 2. (A) Validation of the PAROFF system in different mammalian cell lines. HeLa, HEK-293, hMSC-TERT, HT-1080, BHK-21 and CHO-K1 were co-transfected with pWH8 and pWH10 and cultivated for 48 h in the presence (200 µM) or absence (0 µM) of propylparaben before SEAP levels were profiled in the culture supernatant. Values above the cell line-specific SEAP expression bars indicate the fold repression factor. (B) Validation of the PARON system in different mammalian cell lines. HeLa, HEK-293, HT-1080, hMSC-TERT, BHK-21 and CHO-K1 were co-transfected with pWH9 and pWH5 and cultivated for 48 h in the presence (200 µM) or absence (0 µM) of propylparaben before SEAP levels were profiled in the culture supernatant. Values above the cell line-specific SEAP expression bars indicate the fold induction factor. All data are shown as the mean ± SD, n = 3 independent experiments.
**Figure 3.** Design and characterization of clonal PAROFF- and PARON-transgenic cell lines. (A) Propylparaben-repressible SEAP expression of different PAROFF-transgenic cell clones (HEK-PAROFF). HEK-293 cells were stably co-transfected with pWH8 (PSV40-PMA-pA) and pWH10 (PPMA-SEAP-pA) and six randomly selected cell clones were profiled for their propylparaben-repressible SEAP regulation performance by cultivating them for 48 h in the presence (200 µM) and absence (0 µM) of propylparaben. (B) Dose-dependent SEAP expression profile of the HEK-PAROFF cell line after cultivation for 48 h in the presence of increasing propylparaben concentrations (0–200 µM). (C) SEAP expression kinetics of HEK-PAROFF (2×10^5 cells/ml) cultured for 96 h in culture medium containing increasing concentrations of propylparaben (0–200 µM). Prior to addition of propylparaben (time point 0), HEK-PAROFF cells were cultivated in paraben-free medium. (D) Reversibility of HEK-PAROFF-mediated SEAP expression. 2×10^5 cells/ml HEK-PAROFF cells were cultivated for 144 h while alternating the propylparaben concentrations from 0 to 200 µM and adjusting the cell density to 2×10^5 every 48 h. (E) Propylparaben-inducible SEAP expression of different PARON-transgenic cell clones (HEK-PARON). HEK-293 cells were stably co-transfected with pWH9 (PSV40-PMS-pA) and pWH5 (PPMS-SEAP-pA) and six randomly selected cell clones were profiled for their propylparaben-inducible SEAP regulation performance by cultivating them for 48 h in the presence (200 µM) and absence (0 µM) of propylparaben. (F) Dose-dependent SEAP expression profile of the HEK-PARON6 cell line after cultivation for 48 h in the presence of increasing propylparaben concentrations (0–200 µM). (G) SEAP expression kinetics of HEK-PARON6 (2×10^5 cells/ml) cultured for 96 h in culture medium containing increasing concentrations of propylparaben (0–200 µM). Prior to addition of propylparaben (time point 0), HEK-PARON cells were cultivated in paraben-free medium. (H) Reversibility of HEK-PARON6-mediated SEAP expression. 2×10^5 cells/ml HEK-PARON cells were cultivated for 144 h while alternating the concentrations from 0 to 100 µM and adjusting the cell density to 2×10^5 every 48 h. All data are shown as the mean ± SD, n = 3 independent experiments.

**Cosmetics-controlled transgene expression**

In order to evaluate whether the paraben levels in cosmetics approved by the Personal Care Products Council (<0.8%, (32)) matches the sensitivity range of the PARON system, we exposed pWH9/pWH5-transgenic HeLa cultures to different amounts of commercial skin-care products including toner solutions (cleanser (Lancaster®), skin toner (Lancôme®), emulsion creams (hand cream (Kamill®) and shower gels (Dove®, Cien®)) (Figure 5). Although all of the skin-care products had to be diluted to reduce the cytotoxicity of the soap components, all paraben-containing products were able to dose-dependently induce PARON-driven SEAP expression. Dove® shower gel, which was explicitly declared as paraben-free, was indeed not inducing the PARON device and served as a negative control (Figure 5). The results indicated that the PARON system could be regulated by paraben-containing skin-care and hygiene products in vitro with Lancaster® cleanser and Cien® shower gel showing the best dose-dependent induction performance among all tested toner solutions and emulsions, respectively (Figure 5). However, since the induction performance
Figure 4. Comparative performance analysis of PARON and PEACEON gene switches. (A) Viability of mammalian cells after exposure to different concentrations of phloretin. HEK-293, HeLa and CHO-K1 were cultivated in cell culture medium containing different concentrations of phloretin (0–80 µM) for 48 h before cell viability was scored. (B) Reporter protein-based metabolic integrity assay for phloretin. HEK-293, HeLa and CHO-K1 cells were transfected with the constitutive SEAP expression vector pSEAP2-Control and cultivated in cell culture medium containing different amounts of phloretin (0–80 µM) for 48 h before SEAP levels were profiled in the culture supernatant. (C) Design and functionality of a phloretin-inducible transcription-control system PEACEON. The transsilencer (pMX101, PSV40-TtgS-pA; TtgS, TtgR-KRAB) and the reporter plasmids (pWH19, PSV40-OTtgR2-SEAP-pA) of the PEACEON system are isogenic to the plasmids pWH9 and pWH5 of the PARON system, respectively. Following constitutive expression by the Simian virus 40 promoter (PSV40), TtgS binds and silences a chimeric promoter P_{TtgS} (pWH19, P_{TtgS}-SEAP-pA; P_{TtgS}, PSV40-OTtgR2) containing a tandem TtgS-specific operator module O_{TtgR2} 3’ of a constitutive PSV40. In the absence of phloretin TtgS binds and silences P_{TtgS}-driven SEAP expression, while the presence of phloretin results in the release of TtgS from P_{TtgS} and induces P_{TtgS}-driven SEAP expression. (D) Dose-dependent phloretin-inducible SEAP expression (PEACEON). HEK-293, HeLa and CHO-K1 cells were co-transfected with the PEACEON control components (pMX101/pWH19) and cultivated for 48 h in the presence of increasing phloretin concentrations (0–60 µM) before SEAP levels were profiled in the culture supernatants. (E, F) Control kinetics of the (E) PARON and (F) PEACEON systems. HEK-293 cells were co-transfected with the PARON (pWH9/pWH5) or PEACEON- (pMX101/pWH19) control components and cultivated for 72 h in the presence of increasing concentrations of the corresponding trigger compounds propylparaben (0–200 µM) or phloretin (0–50 µM) before SEAP levels were profiled in the culture supernatants. All data are shown as the mean ± SD, n = 3 independent experiments.
of the cosmetics could be confounded by the cytotoxicity of its soap components for cells grown in culture the true paraben-based control capacity of the cosmetics can only be assessed by percutaneous control of SC PARON-transgenic designer cell implants in an animal model.

**Percutaneous control of SC implants by topical administration of cosmetic skin-care products**

To assess the performance of the PARON system in vivo, we microencapsulated pWH9/pWH5-transgenic HEK-293 cells into coherent, semi-permeable (allowing free diffusion of nutrients, waste metabolites and SEAP) and immunoprotective (pore-size tuned to prevent transfer of immunoglobulins) beads made of alginate-poly-(L-lysine)-alginate, a clinically licensed material that was shown to enable vascularization and connection of entrapped designer cells to the bloodstream (47,48) and has been successfully tested in human-clinical trials (49). Paraben-inducible SEAP expression of microencapsulated PARON-transgenic designer cells was validated in cell culture (Figure 6A) before the same batch was either intraperitoneally (Figure 6B) or subcutaneously implanted into mice (Figure 6C). Animals treated with IP implants received one-dose-per-day of three different concentrations of PP (0–10 mg/kg). Analysis of blood-paraben levels confirmed regulation-effective paraben concentrations in circulation for up to 24 h (60.6 ± 9.1 µM), corroborating established paraben pharmacokinetics (50,51). Forty-eight hours after paraben administration, SEAP expression was profiled in the bloodstream of treated mice (Figure 6B). This data set confirmed dose-dependent high-level performance of the preferred PARON gene switch in vivo yet did not reveal whether the device was sufficiently sensitive to accept percutaneous control input or whether parabens contained in commercial skin-care products would cross the skin to program transgene expression in SC designer cell implants. Therefore, we treated mice with SC PARON-transgenic designer cell implants with three topical applications per day of undiluted Lancaster® cleanser or Kamill® hand cream or solutions containing three different concentrations of PP (0–100 mg/kg) as controls (Figure 6C). Collectively, this data show that paraben (i) is able to cross the skin, (ii) can remotely control cellular behavior in the body in a non-invasive manner simply by topical application of paraben and that (iii) commercial skin-care and hygiene products contain sufficient paraben to program designer cell implants using a typical three-times-per-day application frequency.

**DISCUSSION**

With the ambition to use orthogonal gene switches for the control of therapeutic transgene expression dosing in future gene- and cell-based therapies, the quest for the ideal inducer compounds has just started. While clinically licensed drugs such as antibiotics (13,52–53), hormones (54) and antidiabetics (55) have secondary therapeutic effects and collateral side effects, amino acids (56,57), vitamins (44,58) and metabolites (59) are non-orthogonal and require control concentrations that permanently exceed physiologic levels, food components and food additives such as phloretin (12), preservatives (60) and flavors (60,61) that limit the choice of diet and traceless inducers such as temperature (62,63), light (4) and radiowaves (64) are ubiquitous environmental cues that are impossible to avoid and use for exclusive therapeutic control.

Besides the type of the trigger compound, the administration route will be of prime importance for dosing and...
profiles of microencapsulated PARON-transgenic HEK-293 cells

500 capsules, 200 cells

Figure 6. Validation of the PARON-system in mice. (A) SEAP induction profiles of microencapsulated PARON-transgenic HEK-293 cells in culture. pWH9/pWH5-transgenic microencapsulated HEK-293 cells (1×10⁵ cells, 500 capsules, 200 cells/capsule) were cultivated in 500 μl cell culture medium containing different concentrations of propylparaben (0–200 μM) for 48 h before SEAP levels were profiled in the culture supernatant. The data are shown as the mean ± SD, n = 3. (B) Dose-dependent propylparaben-induced SEAP expression in mice. The same batch of microencapsulated pWH9/pWH5-transgenic HEK-293 cells (2×10⁶ cells, 10 000 capsules, 200 cells/capsule) was intraperitoneally implanted into mice, which received different daily injections of propylparaben (0–10 mg/kg) for 48 h before SEAP levels were quantified in their bloodstream. (C) Percutaneous control of transgene expression in SC implants by topical application of paraben-containing cosmetics. The same batch of microencapsulated pWH9/pWH5-transgenic HEK-293 cells (2×10⁶ cells, 10 000 capsules, 200 cells/capsule) was subcutaneously implanted into mice, which received thrice daily topical applications of paraben-containing cosmetics (three daily administrations: 3×50 μl for a total of 0, 10, 100 mg/kg day⁻¹) as controls. The SEAP levels in the bloodstream of treated animals were profiled after 48 h. The data are shown as the mean ± SEM, statistics by two-tailed t test, n = 8 mice. **P < 0.01, ***P < 0.001 versus control.

patient compliance. In contrast to conventional compound injections, which require medical care, and oral administration, which is limited by the hepatic first-pass effect, transdermal delivery of trigger compounds would improve patient compliance, enable local administration and eliminate the hepatic first-pass effect as well as the need for assistance by trained medical personnel. Pioneering efforts to establish transdermal gene expression control culminated in the design of the phloretin-repressible control element (PEACE), whose expression could be fine-tuned in SC implants by topical application of the penetration enhancer phloretin (12). However, since the PEACE system shows lower trigger sensitivity compared to the paraben control switch in vivo, phloretin needs to be administered at much higher concentrations (1680 mg/kg, (12); compared to 100 mg/kg for PP), which are not present at control-effective levels in off-the-shelf cosmetics.

Collectively, an ideal control compound for therapeutic transgene control should be (i) physiologically inert to prevent any metabolic crosstalk, (ii) clinically licensed to guarantee safety, (iii) rapidly cleared from peripheral circulation to support reversibility of transgene expression and (iv) enable percutaneous control following topical administration.

With their validated generic design principle, their high-performance ON/-OFF-type switch dynamics, combining adjustability and reversibility with low basal as well as high maximum expression profiles, and their responsiveness to the physiologically inert cosmetics preservative PP, the PARON and PAROFF devices meet with all criteria of an ideal gene switch for therapeutic transgene control at a high standard (65). In direct comparison with the ON-type PEACE system providing phloretin-modulated expression control, the isogenic PARON gene switch shows higher induction factors and faster induction kinetics in the tested mammalian cell line. Despite the long history of commonplace application as cosmetics preservative, the finding of increased paraben levels in breast cancer tissue has triggered discussions about the role of parabens in the development of breast cancer (66–68). Although the causal connection has not been scientifically proven and authorities have left the NOAEL (1000 mg/kg day⁻¹) unchanged, an increasing number of consumer product companies feel the consumer pressure and produce paraben-free skin-care products. The PARON system is sufficiently sensitive to reliably test the presence of parabens in cosmetics. For example, using the PARON system we could confirm the presence of parabens in Kamill® hand cream, Lancaster® cleanser and Lancôme Paris® toner as well as the absence of regulation-effective paraben levels in the Dove® shower gel. The paraben-inducible gene switch may therefore also be used to augment animal models for toxicology studies. Furthermore, using paraben-spiked paraben-free commercial skin-care products would allow orthogonal percutaneous remote control of transgenes for therapeutic purposes without interfering with the patients’ lifestyle or hygiene habits. Because of the duty of declaration for parabens in consumer care products and cosmetics, the risk of accidental exposure of individuals to parabens is considered negligible.

Collectively, due to the combination of high skin permeation capacity, low toxicity and a regulation-effective concentration range that fully matches FDA-approved doses

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Collectively, due to the combination of high skin permeation capacity, low toxicity and a regulation-effective concentration range that fully matches FDA-approved doses
(≤0.1%), parabens particularly qualify for remote control of therapeutic transgene expression in SC designer cell implants for safe transdermal therapeutic applications in the future.

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