Cosmetics-triggered percutaneous remote control of transgene expression in mice

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Received November 03, 2014; Revised March 30, 2015; Accepted March 31, 2015

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 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 alkyolated 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 component of the Gram-negative bacterium Pseudomonas syringae pathovar tomato DC3000, a plant pathogen that causes bacterial specks of tomato (38). Expression of P. syringae’s major multidrug efflux pump MexAB-OprM is regulated by PmeR (Pseudomonas multidrug efflux regulator), a TetR-type transcriptional repressor that binds to an inverted repeat (OmeR) overlapping with the promoters driving mexAB-oprM and pmeR (39,40). Parabens...
have been shown to induce the expression of the *mexABC-ompM* genes by binding to PmeR and disrupting the PmeR−O<sub>PmeR</sub> interaction, thereby conferring resistance to multiple plant defense metabolites including parabens (40, 41). Taking advantage of the paraben-responsive PmeR−O<sub>PmeR</sub> 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 ddH<sub>2</sub>O-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) fetal bovine serum (FBS; Sigma-Aldrich, Buchs, Switzerland; cat. no. F7524, lot no. 022M3395) and 1% (v/v) penicillin/streptomycin solution (Biowest, Nuaillé, France; cat. no. L0022-100). Wild-type Chinese hamster ovary cells (CHO-K1, ATCC: CCL-61) were cultured in ChoMaster<sup>®</sup> HTS (Cell Culture Technologies, Gravesano, Germany) supplemented with 5% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. All cell lines were transfected using an optimized polyethyleneimine (PEI)-based protocol (43). In brief, adherent cells cultivated in 24-well plates (50 000 cells in 500 μl medium per well) were incubated with 100 μl of a 1:3 PEI:DNA mixture (w/w) (PEI; MW 40 000, stock solution 1 mg/ml in ddH<sub>2</sub>O; Polysciences, Eppelheim, Germany; cat. no. 24765-2) containing 0.6 μg of total DNA. After 6 h, the culture medium was replaced by 500 μl PEI-free medium containing different concentrations of parabens. Cell concentration and viability were profiled with a CASY<sup>®</sup> Cell Counter and Analyser System Model TT (Roche Diagnostics GmbH, Mannheim, Germany).

**Construction and characterization of stable cell lines**

The HEK-PAR<sub>OFF</sub> 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 pWH9 (P<sub>SV40-PMA</sub>-pA), pWH10 (P<sub>PMA-SEAP-pA</sub>) and pZeoSV2(+)(P<sub>SV40-zeo-pA</sub>) 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-PAR<sub>OFF</sub> was used for all follow-up studies. Likewise, the HEK-PAR<sub>ON</sub> 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 (P<sub>SV40-PMA-pA</sub>), pWH5 (P<sub>PMS-SEAP-pA</sub>) and pZeoSV2(+)(P<sub>SV40-zeo-pA</sub>) 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-PAR<sub>ON</sub> 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 MgCl<sub>2</sub>, 21% diethanolamine, pH 9.8] 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 phlorhizin (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 ddH<sub>2</sub>O). Phlorhizin 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 ddH<sub>2</sub>O). Cien® shower gel (Cien® spring bloom magic shower gel; Lidl Stiftung & Co. KG, Neckarsulm, Germany) and Dove® shower gel (Dove® Duschcheutendezle Pflege; Unilever, Hamburg, Germany) were diluted 100x in 25% DMSO (in ddH<sub>2</sub>O). Lancaster® cleanser (Lancaster® Express Cleanser, Lancaster, Paris, France) and Lancôme Paris® toner (Lancôme Paris® soft-
enabling hydrating toner, L’Oreal, Paris, France) were diluted 10x and 5x in ddH2O, respectively.

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSEAP2-Control</td>
<td>Constitutive mammalian SEAP expression vector (Psv40-SEAP-pA).</td>
<td>Clontech, CA</td>
</tr>
<tr>
<td>pUC57</td>
<td>pUC19-derived prokaryotic expression vector</td>
<td>GeneScript, NJ</td>
</tr>
<tr>
<td>pZeoSV2(+)</td>
<td>Constitutive mammalian expression vector encoding the zeocin resistance gene (Pucmv-zeo-pA).</td>
<td>Invitrogen, CA</td>
</tr>
<tr>
<td>pSAM200</td>
<td>Constitutive mammalian 1TA expression vector (PsV40-1TA-pA).</td>
<td>(14)</td>
</tr>
<tr>
<td>pWW124</td>
<td>γ-butyrolactone (SCB1)-repressible SEAP expression vector (Ppma-SEAP-pA).</td>
<td>(15)</td>
</tr>
<tr>
<td>pMM15</td>
<td>Mammalian expression vector (PsV40-pA).</td>
<td>unpublished</td>
</tr>
<tr>
<td>pMX101</td>
<td>Constitutive mammalian TtgS expression vector (PsV40-TtgS-pA; TtgS, TtgR-KRAB). TtgS was excised from pMG11 using NotI/BsiHII and ligated into the corresponding sites (NotI/BsiHII) of pWW9.</td>
<td>This work</td>
</tr>
<tr>
<td>pWH1</td>
<td>pUC57 containing a custom-designed mammalian codon-optimized PmeR</td>
<td>This work</td>
</tr>
<tr>
<td>pWH5</td>
<td>Paraben-inducible SEAP expression vector (Ppms-SEAP-pA; Ppms, Psv40-Popm2).</td>
<td>This work</td>
</tr>
<tr>
<td>pWH8</td>
<td>Constitutive PMA expression vector (Ppsv40-PMA-pA; Ppma, Pmer-vp16).</td>
<td>This work</td>
</tr>
<tr>
<td>pWH9</td>
<td>Constitutive PMS expression vector (Ppsv40-PMS-pA; Ppms, Pmer-KRAB).</td>
<td>This work</td>
</tr>
<tr>
<td>pWH10</td>
<td>Paraben-repressible SEAP expression vector (Ppma-SEAP-pA; Ppmas, Opmod-2; Ppmm2). OPmod2 was excised from pWH5 using AatII/SbfI and ligated into the corresponding sites (AatII/SbfI) of pWW124.</td>
<td>This work</td>
</tr>
<tr>
<td>pWH19</td>
<td>Phloretin-inducible SEAP expression vector (Ptgg-SEAP-pA; PtggS, Psv40-Ttg2).</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Oligonucleotides:** Restriction endonuclease-specific sites are underlined, annealing base pairs are indicated in capital letters and the operator module O pmeR is shown in bold. **Abbreviations:** KRAB, Krueppel-associated box protein of the human kox-I gene; KstR, Mycobacterium tuberculosis repressor of the cholesterol biosynthesis; Opap1, SCA-specific operator; Opmod2, tandem PmeR-specific operator; Opapp2, tandem PtgR-specific operator; pA, polyadenylation site; PmeR, repressor of the Pseudomonas syringae pathovar tomato DC3000-driven multidrug efflux pump; PsVMV, human cytomegalovirus immediate early promoter; PsCMVMin, minimal version of PsCMV; PMA, PmeR-derived paraben-dependent transactivator (PmeR-KRAB); PSPA, γ-butyrolactone (SCB1)-repressible promoter (Opap2-Ppms-SEAP-pA); Ppma, paraben-repressible promoter (Opapp2-Ppmm2); Ppsv40, paraben-inducible promoter (Ppsv40-Popm2); PsV40, simian virus 40 promoter; PtggS, phloretin-inducible promoter (PsV40-Ptgg2); SCA, SCB1-dependent transactivator (Scb1-VP16); Scb1, Streptomyces coelicolor 1,1-(β-D-glucopyranoside) (SCB1)-specific quorum-sensing receptor; SEAP, human placental secreted alkaline phosphatase; TetR, Escherichia coli TdR-dependent tetacycline-dependent repressor of the tetracycline resistance gene; ITA, tetracycline-dependent transactivator (TetR-VP16); TtgA1, phloretin-dependent transactivator (TtgR-VP16); TtgR, repressor of the Pseudomonas putida DOT-T1E ABC multidrug efflux pump; TtgS, TtgR-derived phloretin-dependent transsenser (TtgR-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 Labotechnik 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 SC (lower dorsum) implantation of 1×10⁶ microcapsules into eight-week-old female OF1 mice (onsins France souche 1; Charles River Laboratories, France), the animals were treated with PP injections (0–10 mg/kg in 50 μl DMSO, once every 24 h) or topical application of commercial hand cream (Kamill®, 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, collected blood samples after 24 h and adding 10 μl of serum to 2.5x 10⁴ 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}:

- Parabens
  \[
  \begin{array}{c}
  \text{SEAP} \\
  \text{ON}
  \end{array}
  \]

+ Parabens
  \[
  \begin{array}{c}
  \text{SEAP} \\
  \text{OFF}
  \end{array}
  \]

Regulation of PAR\textsubscript{ON}:

- Parabens
  \[
  \begin{array}{c}
  \text{SEAP} \\
  \text{OFF}
  \end{array}
  \]

+ Parabens
  \[
  \begin{array}{c}
  \text{SEAP} \\
  \text{ON}
  \end{array}
  \]
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 P_{PMA} (pWH10, P_{PMA}-SEAP-pA; P_{PMA}, O_{PmeR2}-PhCMVmin) containing a tandem PMA-specific operator module O_{PmeR2} 5' of PhCMVmin, which is set to drive expression of SEAP. In the absence of parabens PMA binds P_{PMA} and drives SEAP expression, while the presence of paraben derivatives results in the release of PMA from O_{PmeR2} 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-1 gene. Following constitutive expression by pSV40, PMS binds and silences a chimeric promoter P_{PMS} (pWH5, P_{PMS}-SEAP-pA; P_{PMS}, PSV40-O_{PmeR2}) containing a tandem PMS-specific operator module O_{PmeR2} 3' of a constitutive PSV40. In the absence of parabens PMS binds and silences P_{PMS}-driven SEAP expression, while the presence of paraben derivatives results in the release of PMS from P_{PMS} and induces P_{PMS}-driven SEAP expression.

(C) Reporter protein-based metabolic integrity assay and (D) cell proliferation assay of PMA- and PMS-expressing cells. HEK-293 cellswere 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 viiability was scored. (F) Reporter protein-based metabolic integrity assay for paraben derivatives. HEK-293 cells were co-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) before SEAP levels were profiled in the culture supernatant. Repression factors: MP: 3; 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 interplay of synthetic PPMA promoters (pWH10, PPMA-SEAP-transactivation domain (VP16), that modulates the activity of synthetic PPMS promoters (pWH5, (KRAB) domain of the human cytomegalovirus immediate-early promoter (Pκ) in a paraben-responsive manner: In the absence of parabens, PmeR binds and activates PPMA-driven transgene expression while paraben derivatives prevent the inhibition (Figure 1A).

PAROFF consists of the synthetic mammalian transcription factor PMA (paraben-mediated transactivator; pH8, PSV40-PPMA-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 PPMAs promoters (PWH10, PPMAS-SEAP-pA; PPMAS, OPmeR2-PκCMVmin), containing the PMA-specific tandem operator module OPmeR2 5’ of a minimal version of the human cytoskeletal virus immediate-early promoter (PκCMVmin), in a paraben-responsive manner: In the absence of parabens, PMA binds and activates PPMA-driven transgene expression while paraben derivatives prevent the PAROFF system, by stably co-transfecting pWH8 and pWH10 into HEK-293. All of these transgenic HEK-293 cell lines showed functional PP-inducible expression control (Figure 1F).

PARON consists of a synthetic mammalian transcription silencer PMS (paraben-mediated transsilencer; pH9, PSV40-PMS-pA; PMS, PmeR-KRAB), engineered by fusing PmeR’s C-terminus to the Krueppel-associated box (KRAB) domain of the human kox-1 gene, that modulates the activity of synthetic PPMAs promoters (PWH5, PPMAS-SEAP-pA; PPMAS, PSV40-OPmeR2), containing the PMS-specific tandem operator module OPmeR2 3’ of the constitutive simian virus 40 promoter (Pκ40), in a paraben-responsive manner. In the absence of parabens PMS binds and represses PPMS while paraben derivatives prevent the PARON system toowas functional (Figure 1F).

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

We have generated six clonal double-transgenic cell lines (HEK-PAROFF-4), in which SEAP is controlled by the PAROFF system, by stably co-transfecting pH8 and pH10 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-PARON emerged as the best in-class transgenic cell line that was chosen for all follow-up studies. Also, HEK-PAROFF 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-PARON 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; pWH19, PSV40-OTtgR2-SEAP-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 pWH9 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 (PMA-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) cultivated 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 (PMS-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-PARON cell line after cultivation for 48 h in the presence of increasing propylparaben concentrations (0–200 µM). (G) SEAP expression kinetics of HEK-PARON (2 × 10^5 cells/ml) cultivated 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-PARON-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 PSAP2-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 PtgS (psV40-SEAP-pA; PtgS, PSV40-OTtgR2) containing a tandem TtgS-specific operator module OtgR2 3' of a constitutive Psv40. In the absence of phloretin TtgS binds and silences PtgS-driven SEAP expression, while the presence of phloretin results in the release of TtgS from PtgS and induces PtgS-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.
Figure 5. Cosmetics-induced PARON-dependent SEAP expression in vitro. HeLa cells were co-transfected with pWH9 and pWH5 and cultivated in cell culture medium containing different cosmetic products (Dove® shower gel, Kamill® hand-cream, Lancôme Paris® Tonic, Cien® shower gel, Lancaster® Express Cleanser) at different dilutions (v/v) or different concentrations (0–60 µM) of various parabens (MP, methylparaben; EP, ethylparaben; PP, propylparaben) (insert). Addition of the DMSO was used as solvent control. Forty-eight hours after addition of cosmetics, SEAP levels in the culture supernatant were profiled. 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) as controls (Figure 6C). Animals treated with IP implants received one-dose-per-day of three different concentrations of PP (0–10 mg/kg) as controls (Figure 6C). Animals treated with IP implants received one-dose-per-day of three different concentrations of PP (0–10 mg/kg) as controls (Figure 6C). Animals treated with IP implants received one-dose-per-day of three different concentrations of PP (0–10 mg/kg) as controls (Figure 6C). Animals treated with IP implants received one-dose-per-day of three different concentrations of PP (0–10 mg/kg) as controls (Figure 6C). Animals treated with IP implants received one-dose-per-day of three different concentrations of PP (0–10 mg/kg) as controls (Figure 6C). Animals treated with IP implants received one-dose-per-day of three different concentrations of PP (0–10 mg/kg) as controls (Figure 6C). Animals treated with IP implants received one-dose-per-day of three different concentrations of PP (0–10 mg/kg) as controls (Figure 6C). Animals treated with IP implants received one-dose-per-day of three different concentrations of PP (0–10 mg/kg) as controls (Figure 6C).

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 radio waves (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
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 (1×10^5 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 (2×10^6 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 (2×10^6 cells, 10 000 capsules, 200 cells/capsule) was subcutaneously implanted into mice, which received three daily topical applications of paraben-containing cosmetics (three daily administrations: 3×300 µl Kamill® hand cream; 3×300 µl Lancaster® cleanser). Solutions containing different concentrations of propylparaben (three daily administrations: 3×50 µl for a total of 0, 10, 100 mg/kg day^-1) were used 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.
(<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.

ACKNOWLEDGEMENTS
We thank Lina Schukur, Pratik Saxena and Peng Bai for generous advice, Katrin Roessler for providing pKR71, Marius Mueller for providing pMM15 and Ghislaine Charpin-El-Hamri for support in the animal study.

FUNDING
ERC advanced (321381); INTERREG IV A.20 (in part); Gutenberg Chair awarded (to M.F.). Funding for open access charge: ERC - Advanced Grant.

Conflict of interest statement.

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