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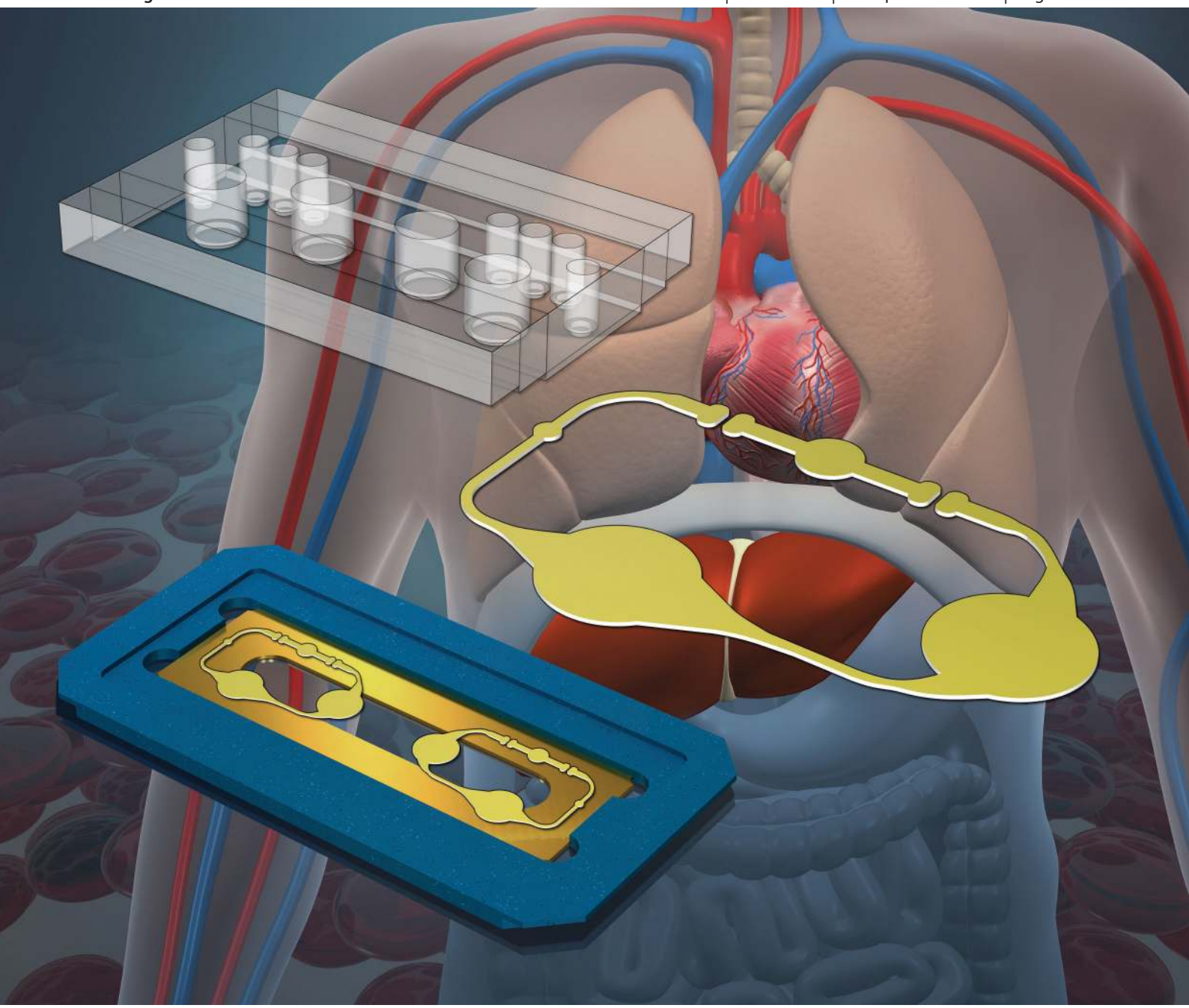
Volume 13 | Number 18 | 21 September 2013 | Pages 3435–3766



ISSN 1473-0197

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Cite this: *Lab Chip*, 2013, **13**, 3538

A dynamic multi-organ-chip for long-term cultivation and substance testing proven by 3D human liver and skin tissue co-culture

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Received 20th February 2013,
Accepted 28th March 2013

DOI: 10.1039/c3lc50234a

www.rsc.org/loc

Current *in vitro* and animal tests for drug development are failing to emulate the systemic organ complexity of the human body and, therefore, to accurately predict drug toxicity. In this study, we present a multi-organ-chip capable of maintaining 3D tissues derived from cell lines, primary cells and biopsies of various human organs. We designed a multi-organ-chip with co-cultures of human artificial liver microtissues and skin biopsies, each a $^{1}/_{100\,000}$ of the biomass of their original human organ counterparts, and have successfully proven its long-term performance. The system supports two different culture modes: i) tissue exposed to the fluid flow, or ii) tissue shielded from the underlying fluid flow by standard Transwell® cultures. Crosstalk between the two tissues was observed in 14-day co-cultures exposed to fluid flow. Applying the same culture mode, liver microtissues showed sensitivity at different molecular levels to the toxic substance troglitazone during a 6-day exposure. Finally, an astonishingly stable long-term performance of the Transwell®-based co-cultures could be observed over a 28-day period. This mode facilitates exposure of skin at the air–liquid interface. Thus, we provide here a potential new tool for systemic substance testing.

Introduction

New solutions are needed to ban the ever increasing dilemma of poorly predictive preclinical substance evaluation generated by the phylogenetic distance between laboratory animals and humans, the discrepancy between current *in vitro* systems and the human body, and the restrictions of *in silico* modelling. Although it is an ambitious project, the development of ''human-on-a-chip'' systems has been recognised as an ideal path to escape from this dilemma.[1–3] These ''human-on-a-chip'' systems combine two essential requirements: i) the improvement of the organotypical nature of single organ equivalents, and ii) the linking of different organs into a systemic arrangement reflecting that of the human organism at a miniaturised scale. Only a few multi-compartment cell culture flow systems for the simultaneous co-culture of different tissues have been described on a miniaturised scale so far.[4–7] The majority expose tissues to laminar flow within microchannels.[3,5,8] The advantage of these multi-organ systems is an explicit adjustable fluid flow and a controllable local tissue-to-fluid ratio in the channels. However, these systems suffer from reduced cross-conditioning and molecular crosstalk among tissues, due to a small cell count (mostly below ten thousand) and a significant dilution. Tissue crosstalk has been reported by Guzzardi *et al.*[11] in a system hosting 2.4×10^5 liver cells and 8×10^4 endothelial cells in separate compartments with a total circulating volume of 30 ml medium. Tissue volumes in those systems represent only a very tiny fraction of the overall circulating media volume due to the use of external pumps and external media reservoirs.

To solve this problem, we integrated a peristaltic micropump and media reservoirs into a chip the area of a standard microscope slide, in order to minimise the fluid-to-tissue ratio within the whole system and to ensure a selectable number of media flow velocities. This multi-organ-chip (MOC) combines the on-chip micropump with various arrangements of separate standardised tissue culture spaces connected by microfluidic channels. Of several designs produced, the two-tissue MOC design was chosen to prove the capability of the MOC platform to simultaneously maintain a human liver equivalent and a human skin biopsy. The liver is the prime target for toxicity

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evaluation of pharmaceutical drug candidates and human skin is the target tissue for cosmetics. The human hepatocyte cell line, HepaRG, primary human hepatic stellate cells (HHSteC) and freshly generated skin biopsies were cultured simultaneously to demonstrate the comprehensiveness of the MOC system with regard to tissue sources. Each of the perfused MOC tissue culture compartments was designed the scale of a single well of a standard 96-well plate to fit industrially applied standard formats of *in vitro* culture. This allowed the direct exposure of co-cultures to fluid flow at the bottom of the compartments and the co-culture to be shielded from the underlying fluid flow by standard Transwell® inserts. The latter mode, in addition, facilitates the culture of skin at the air–liquid interface. Furthermore, the MOC device's stable long-term performance enables repeated dose exposure of tissues to troglitazone, a diabetic drug with well-known dose-dependent liver toxicity.

Materials and methods

Device design and fabrication

We established a rapid prototyping procedure for the flexible fabrication of various designs of MOCs. A single 2 mm high polydimethylsiloxane (PDMS) layer containing the respective arrangement of channels, micropumps and openings for culture compartments (Fig. 1a) was permanently bonded by low pressure plasma oxidation (Femto; Diener, Ebhausen, Germany) to a glass microscope slide with a footprint of 75 × 25 mm (Menzel, Braunschweig, Germany), thus forming the respective fluid-tight microfluidic MOC device with standard channel heights of 100 μm. The peristaltic on-chip micropump was modified from Wu and co-workers[9] and represents three 500 μm thick PDMS membranes in a row. They are consecutively actuated by applying pressure. Up to seven tissue culture compartments have been designed into MOC devices for different applications (larger black areas of each design in Fig. 1a). The design consisting of two culture compartments per co-culture circuit (Fig. 1a, top image) was used in the experiments reported here. The MOCs were fabricated applying standard soft lithography and replica moulding of PDMS (Sylgard 184, Dow Corning, Midland, MI, USA). In brief, a master mould was fabricated by bonding a silicon wafer to a glass wafer. Photoresist was applied to the silicon wafer and patterned by using a photomask and UV light. Subsequently, unprotected silicon regions were etched and the photoresist was stripped. The fabrication of the microsystem was as follows: A polycarbonate cover-plate was treated with a silicon rubber additive (WACKER® PRIMER G 790; Wacker Chemie, Munich, Germany) at 80 °C for 20 min. Teflon screws were inserted to create four PDSM-free compartments for culture and six 500-μm thick PDMS membranes to connect the micropumps. The prepared cover-plate was plugged to the master mould (channel height 100 μm, width 500 μm) and PDMS (10 : 1 v/v ratio of PDMS to curing agent) was injected into this casting station. The set-up was incubated at 80 °C for 60 min and, subsequently, bonded to the glass slide. A schematic drawing of the final MOC device is illustrated in Fig. 1b and highlights the fluid flow directions. Fig. 1c illustrates the culture compartments, each designed to hold a maximum of 300 μl medium. The direct exposure of

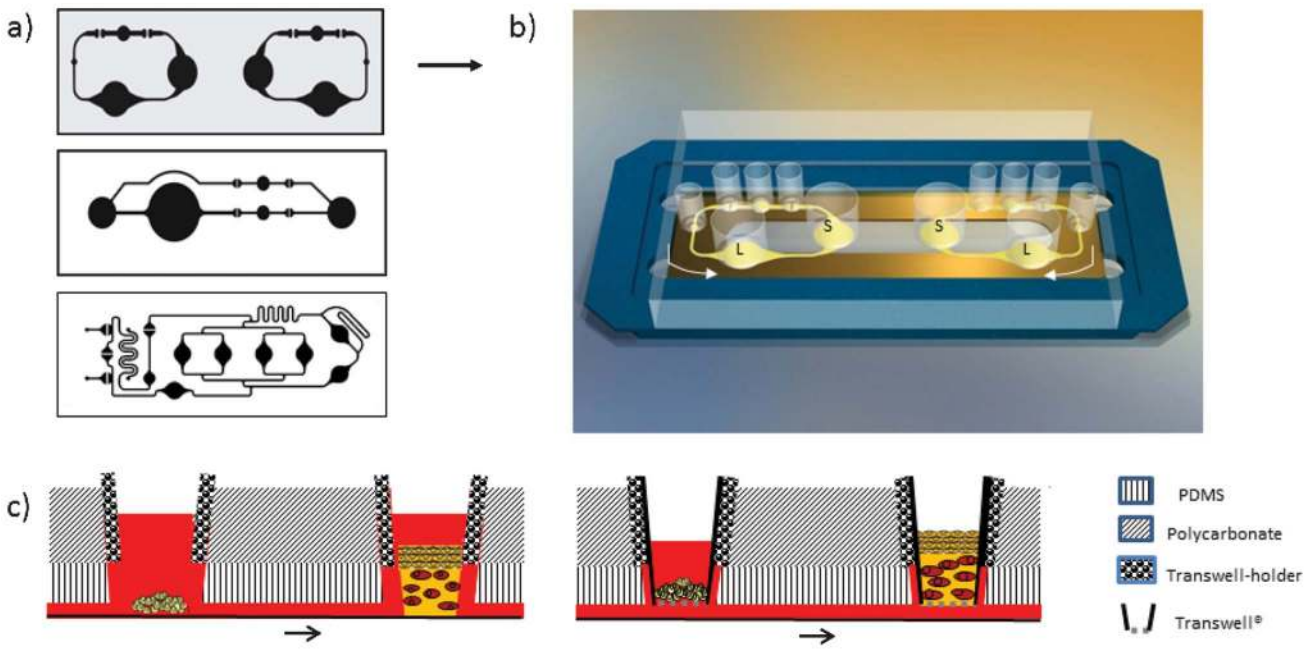

Fig. 1 The microfluidic MOC device at a glance. a) Bird's-eye view of three different MOC designs. b) 3D drawing of an assembled MOC with two microfluidic circuits in a PDMS-glass chip attached to a cover-plate and placed into a MOC holder (blue). Arrows indicate fluid flow direction of each circuit. S-skin culture compartments, L-liver culture compartments c) Schematic sections through the tissue culture compartments supporting both submersed tissue cultures in the fluid flow (left image) or cultures in Transwell® (right image).

tissue to fluid flow (left side) and the alternative co-culture setting in Transwell® separating tissues from the fluid flow by a 10 μm porous membrane (right side) are highlighted.

Cell sources and maintenance

Cell culture components were purchased from PAA Laboratories (GE Healthcare Europe GmbH, Vienna, Austria) and cultures were incubated in HepaRG medium at 37 °C and 5% CO_2, unless otherwise stated. HepaRG cells were obtained from Biopredic International (Rennes, France) and maintained as described by Gripon *et al.*[10] Briefly, cells were cultured in HepaRG medium, consisting of William's Medium E supplemented with 10% foetal calf serum, 100 units per ml penicillin, 100 μg ml^{-1} streptomycin, 5 μg ml^{-1} human insulin, 2 mM L-glutamine, and 5×10^{-5} M hydrocortisone hemisuccinate (Sigma-Aldrich, St. Louis, MO, USA). Undifferentiated cells were maintained in 75 cm^2 tissue culture flasks (Greiner Bio One, Germany) at a seeding density of 2×10^4 cells cm^{-2} for two weeks, according to the manufacturer's instructions. Medium was renewed every 2 or 3 days. Induction of differentiation was initiated by maintaining the cells in growth medium for 2 weeks with a regular media exchange every 2 or 3 days in order to reach cellular confluence. Differentiation medium containing 2% dimethyl sulfoxide (DMSO; Carl Roth GmbH, Karlsruhe, Germany) was then added for a cultivation period of 2 weeks. When differentiation was completed, the cells were used for further experiments. Human hepatic stellate cells (HHSteC) and their culture supplements were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were seeded at 5×10^3 cells cm^{-2} in poly-L-lysine-coated 75 cm^2 tissue culture flasks in stellate cell medium for maintenance, according to the manufacturer's instructions. Medium was exchanged every 2 or 3 days. Cells were harvested for further use at 90% confluence. Human juvenile prepuce was obtained in compliance with the relevant laws, with informed consent and ethics approval (Ethic Committee Charité University Medicine, Berlin, Germany), from a paediatric surgery after routine circumcisions. Prepuce samples with an average size of 2.5 cm^2 were stored and transported in 10 ml phosphate-buffered saline (PBS) at 4 °C and prepared for further culture within 4 h following surgery.

Preparation of skin biopsies

Prepuce samples were sterilised in 80% ethanol for 30 s. Samples with an average height of 2 mm were punched to biopsies with 4 or 5 mm diameter, depending on the culture set-up chosen. One biopsy was loaded into a single MOC culture compartment for cultivation. Samples from one donor were used for each MOC-based test series and the respective control in static culture (punches from Stusche, Teltow, Germany).

Formation of human artificial liver microtissues

Liver microtissue aggregates were formed in Perfecta3D® 384-Well Hanging Drop Plates (3D Biomatrix, Ann Arbor, MI, USA), according to the manufacturer's instructions. Briefly, 20 μl cell suspension containing 4.8×10^4 hepatocytes and 0.2×10^4 HHSteC were pipetted to each access hole. After two days of hanging drop culture, aggregates were transferred to ultra-low attachment 24-well plates (Corning, Lowell, MA, USA) with a maximum of 20 aggregates per well. Following culture in ultra-low attachment wells for 3 days, 20 aggregates were loaded into a single tissue culture compartment of the MOC.

Multi-tissue culture directly exposed to fluid flow in the MOC device

Liver microtissues (20 aggregates per compartment) and a 4 mm skin biopsy (one biopsy per compartment) were loaded into the respective culture compartment of the two circuits of each MOC device (Fig. 1b) for co-culture. Additional MOCs were loaded solely with liver microtissue and solely with skin biopsies for comparison. Subsequently, 300 μl of medium was added to each culture compartment, which was then hermetically sealed by a lid. During the first 7 days, a 40% media exchange rate was applied at 12 h intervals. From day 7 onwards, a 40% exchange rate was applied at 24 h intervals. Daily samples were collected for respective analyses. Experiments were stopped at day 14 of co-culture and tissues of the MOCs were subjected to immunohistochemical stainings. Experiments were conducted with six replicates.

Multi-tissue exposure to troglitazone

Troglitazone was dissolved in DMSO, stored frozen at a concentration of 20 mM until use, and then diluted in culture medium to a level of 0.05% DMSO. Medium containing 0.05% DMSO was used for control cultures. Liver microtissue and skin biopsy co-cultures in MOCs were prepared, cultured and analysed as described above. The contents in the MOCs were cultured for one day prior to exposure and were, subsequently, exposed to 0 μM, 5 μM and 50 μM troglitazone, respectively. Application of troglitazone was repeated at 12 h intervals simultaneously with the medium change. The experiments were stopped at day 7. Cell viability and protein expression were assessed by immunohistochemistry and quantitative PCR (qPCR). Experiments were performed in quadruplicate.

Transwell® multi-tissue culture in the MOC device

Liver microtissues and skin biopsies were cultured for cosmetic compliance each in a separated single insert of a 96-well Transwell® unit (Corning), which was hung inside the chip with the membrane fitting directly over the circuit. The height of the tissue and the amount of medium added to the Transwell® enabled the tissue to be either air–liquid interfaced (skin) or submerged in media (liver equivalent). Compartments for tissue culture were filled with 200 μl medium each to inoculate the MOC. Two wells per circuit from a 96-well Transwell® plate (0.4 μm pore size) were cut below the bracket with an incandescent knife and removed. Prepared liver spheroids and epidermal-side up skin tissues with a diameter of 5 mm were transferred into the excised Transwell® which were positioned into the transwell-holders and screwed into the MOC. The medium was exchanged every 12 h within the first 7 days and every 24 h afterwards, as described above. Supernatants were collected every day for lactate and glucose measurements. At the end of the 28-day culture, cell viability and protein expression were assessed *via*

immunohistochemistry. The experiments were conducted in quadruplicate.

Tissue culture analyses

Tissue viability was monitored daily by the measurement of lactate dehydrogenase (LDH) released in the supernatant. In brief, samples were immediately measured for LDH concentration and then stored at −80 °C for further analysis of glucose, lactate and albumin. All absorbance-related measurements were performed in 96-well microtitre-plates (Greiner Bio-One, Frickenhausen, Germany) in a microplate-reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany), if not stated otherwise. The LDH concentration of the medium was measured using the LDH Liqui-UV kit (Stanbio Laboratory, Boerne, USA), according to the manufacturer's instructions with minor modifications based on our own standard curves: An amount of 100 μl of reagent was used and 1 μl of the sample was added for each measurement.

Furthermore, tissue behaviour, apoptosis and proliferation were analysed by immunohistological end-point stainings at the end of each MOC experiment using TUNEL (TdT-mediated dUTP-digoxigenin nick end labelling)/Ki67 markers. Briefly, representative central sections of the tissue of each sample were selected for staining. Eight-micron cryostat sections per sample of skin and liver were stained for apoptosis using the TUNEL technique (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. The apoptosis staining was combined with a nuclear stain, Hoechst 33342 (Life Technologies, Darmstadt, Germany), and, where sections were also stained for proliferation, an antibody Ki67 (Abcam, Cambridge, UK). The sections were fixed in acetone at −20 °C for 10 min, washed with PBS and blocked with 10% goat serum in PBS for 20 min, then incubated with rabbit anti-human Ki67 antibody at 4 °C overnight. The sections were washed and incubated with secondary antibody goat anti-rabbit IgG Alexa Fluor® 594 (Life Technologies) for 45 min, washed, incubated with Hoechst 33342 dye (10 μg ml^{-1} in PBS) for 10 min and washed again before fluorescence imaging.

Glucose consumption, lactate production and albumin synthesis was monitored daily to assess the metabolic activity of the tissues in the MOC. The Glucose LiquiColor® Oxidase kit (Stanbio Laboratory, Boerne, USA) was used with minor modifications: An amount of 100 μl of reagent was used and 1 μl of sample was added. Lactate concentration of the medium was measured using the LAC 142 kit (Diaglobal, Berlin, Germany), according to the manufacturer's instructions with minor modifications based on our own standard curves: An amount of 99 μl of the reagent was mixed with 1 μl of sample and absorbance was measured at 520 nm, using medium as a reference. The Human Albumin ELISA Quantitation Set (Bethyl Laboratories, Montgomery, USA) was used to measure the albumin concentration, according to the manufacturer's instructions.

Real-time qPCR endpoint analyses were performed to evaluate liver gene transcription at mRNA level after the MOC cultures were stopped as follows: Liver equivalents were collected for RNA isolation using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesised by reverse transcription of 400 ng total RNA (TaqMan®, Roche Diagnostics, Mannheim, Germany). Real-time qPCR experiments were conducted by using the Stratagene system (Agilent Technologies, Böblingen, Germany) and the SensiFast SYBR No-ROX One-Step Kit (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions. The real-time qPCR primers were as follows: cytochrome P450 3A4 forward; 5′-GGAAGTGGACCCAGAAACTGC and reverse 5′-TTACGGTGCCATCCCTTGAC, Housekeeping gene: TBP (TATA box binding protein) forward 5′ CCTTG-TGCTCACCCACCAAC 3′ and reverse 5′TCGTCTTCCTGAAT-CCCTTTAGAATAG 3′.

Finally, in-depth immunohistochemical end-point analyses were performed by staining the liver tissue sections for the functional markers cytochrome P450 3A4 and 7A1, the tight junction associated protein ZO-1 and the transporter MRP2, and for the structural proteins cytokeratin 8/18 and vimentin. Skin tissue sections were stained for the epidermal structural proteins cytokeratin 10 and 15. Briefly, representative central cryosections of the tissues were fixed and blocked following the same methods as described for the Ki67 staining. Skin sections were then incubated with mouse anti-human Cytokeratin 10 (Millipore, Billerica, USA) and rabbit anti-human Cytokeratin 15 (Abcam, Cambridge, UK) antibody for 2 h and washed with PBS. Subsequently, goat anti-rabbit IgG Alexa Fluor® 594 (Life Technologies) and goat anti-mouse IgG fluorescein isothiocyanate (FITC) (BD Biosciences, Heidelberg, Germany) conjugated secondary antibodies and Hoechst 33342 were added, and the sections were incubated for 45 min before fluorescence imaging. The same procedure was carried out for liver equivalents using mouse anti-human cytochrome P450 3A4, rabbit anti-human cytochrome P450 7A1, rabbit anti-human ZO-1, rabbit anti-human MRP2, mouse anti-human cytokeratin 8/18, and rabbit anti-human vimentin (all antibodies purchased from Santa Cruz Biotechnology, Heidelberg, Germany). Conjugated secondary antibodies goat anti-mouse IgG Alexa Fluor® 594, goat anti-rabbit IgG Alexa Fluor® 594 and goat anti-rabbit IgG Alexa Fluor® 488 (all purchased from Life Technologies) were used for visualisation.

Results and discussion

Features of the MOC design

The newly designed MOC platform presented here combines a flexible microfluidic channel system (100 μm high) operated by an on-chip micropump with multiple separate tissue culture compartments, each the size of one cavity of a standard 96-well microplate. The design aims to provide two important physiological features: mechanical coupling of tissues and their molecular crosstalk with each other. The standard tissue culture format easily enables researchers to combine validated static tissue cultures of different origin within a common fluid flow (*e.g.* human 3D liver spheroids, Transwell®-based human epidermis and CaCo2-cell barrier models). We have prototyped different MOC designs with up to seven separate tissue culture compartments (Fig. 1a) for various applications so far. Data in this paper are acquired with the microfluidic MOC device (Fig. 1b) consisting of two

separate circuits each containing two tissue culture compartments. Two alternative culture modes with and without use of a Transwell® insert are applicable in each culture compartment (Fig. 1c). The tissue volume cultivated in the system is 26 μl per circuit for the liver and skin tissue co-cultures. The total extra-cellular fluid volume is 600 μl per circuit without the Transwell® and 400 μl per circuit using the Transwell® insert in each compartment, whereof the channel connecting the two tissues (equivalent to intra-capillary fluid volume) has a volume of 10 μl. This corresponds to a total systemic media-to-tissue ratio of 23 : 1 or 15 : 1, respectively, for the liver and skin tissue co-cultures. In humans, regarding a 73 kg man, the total extra-cellular fluid volume is 14.6 l, whereof the inter-capillary fluid volume is 5.1 l. Therefore, the physiological extracellular fluid to tissue ratio would be 1 : 4. In the MOC, the excess amount of media is due to the media demand of the tissue. The micropump provides a stable pulsatile fluid flow at a circulation rate of 40 μl min^{-1} through the microchannel system supporting a total volume perfusion in 10 min (with Transwell® inserts) or 15 min (without Transwell® inserts).

The mechanical coupling of tissues has been shown to be an important physiological factor for functional tissue behaviour in almost all organs at various degrees.[7] Domansky and colleagues adopted mechanical coupling to a multiwell plate for 3D liver tissue engineering. Inside the bioreactors, a scaffold made of an ECM-coated polymer with an array of 769 microchannels (0.24 mm depth, 0.34 mm diameter) was supported below by a filter with 5 μm pore size. Approximately 10^6 cells of a hepatocyte–sinusoidal endothelial cell co-culture were perfused with a total volume of 3 ml media per bioreactor.[12] Unfortunately, this system does not provide the option to combine two or more separate tissue culture compartments. In contrast to the devices described above, our MOC system provides a low systemic media-to-tissue ratio with the flexibility to combine different standard tissue cultures. In addition to the liver and skin tissue co-culture reported here, the combination of, for example, commercial Transwell® barrier models of intestine with submersed liver microtissue cultures is envisaged. This might support further investigations into systemic organ interactions for substance exposure. Furthermore, it might be of interest to study skin permeability of cosmetics with a representative number of perfused human epidermal skin equivalents in respective arrangements.

Robustness of tissue preparation

Skin biopsy preparation was established for more than 30 biopsies with 100% nondissipative and contamination-free preparations and applications within one year. Biopsies of 4 and 5 mm diameter – different sizes for technical use – were prepared, representing $^1/_{100\,000}$ of the human skin area. A 5 mm diameter biopsy has a tissue volume of 24 μl and consists of approximately five million cells. Unlike commercially available skin models, the prepuce contains apocrine glands, which produce lysozymes, neutrophil elastases, and cytokines.[13] In addition, it has numerous specialised nerve endings *in vivo*. Furthermore, adult stem cells, which are capable of regenerating the epidermis, are present in their specific quiescent-promoting niches in the prepuces. This heterogeneity of the original tissue supports different cellular and molecular events in the respective biopsies. Donor-specific variations may explain the high variances for certain data points.

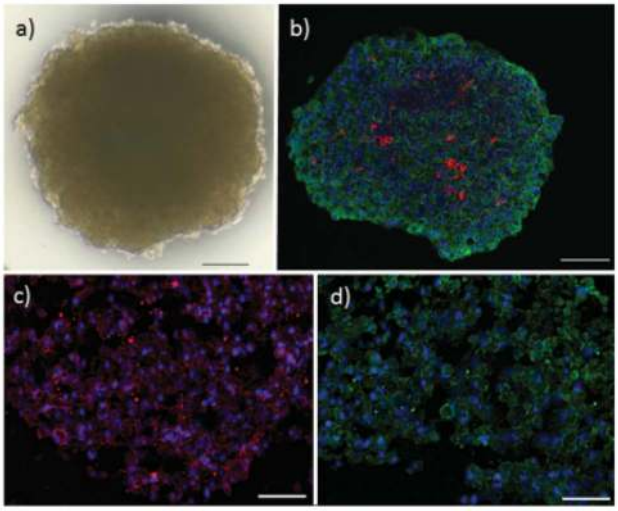

Fig. 2 Formation of human artificial liver microtissues over 2 days. a) Light microscopy of a human liver spheroid generated by a standardised hanging drop culture. Scale bar: 100 μm. b) Equal distribution of HHSteCs throughout the HepaRG cell aggregate at a 1 : 24 ratio demonstrated by immunostaining of vimentin (red) and cytokeratin 8/18 (green), blue nuclear staining. Scale bar 100 μm. c) Characteristic formation of tight junctions (protein ZO-1, red), blue nuclear staining. Scale bar 50 μm. d) Expression of canalicular transporter MRP2 (green), blue nuclear staining. Scale bar 50 μm.

Consistent disk-shaped liver cell aggregates with a medium diameter of 300–400 μm and a height of 200–300 μm were formed during 2 days of hanging drop culture (Fig. 2a). A reliable production of 300 spheres per plate was achieved. One tissue culture compartment of each MOC circuit was seeded with 10^6 cells corresponding to 20 aggregates, which matches an equal miniaturisation ratio for both skin and liver. Immunohistochemical stainings of vimentin and cytokeratin 8/18 revealed that the HHSteCs were distributed equally throughout the whole aggregate (Fig. 2b). ZO-1 staining showed that cell–cell contact supports tight junctions developed during culture (Fig. 2c). Positive staining for MRP-2 demonstrates the polarisation of cells and the existence of rudimentary bile canaliculi-like networks in the generated liver microtissues (Fig. 2d).

Performance of fluid flow exposed multi-tissue cultures over 14 days

The human liver and skin tissue co-cultures were maintained in all MOCs during the experimental period of 14 days (Fig. 3). A constant LDH level of about 20 U L^{-1} from cultivation day 6 onwards indicates an artificial but stable tissue turnover in the system. Cell death within this steady state happened primarily in the skin culture compartment, as data from MOC single-tissue cultures of skin suggest (data not shown). A possible explanation might be an increased epidermis turnover induced by the artificial culture conditions, such as artificial shear stress and lack of air exposure to the epidermal surface of skin biopsies. It is interesting that this steady tissue

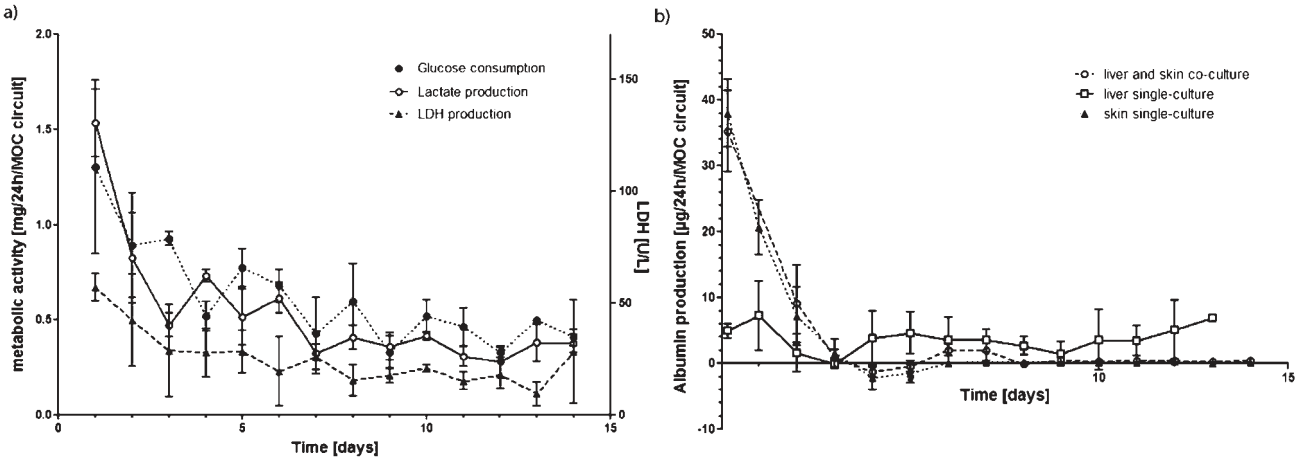

Fig. 3 14-day tissue performance at direct exposure to fluid flow. a) Glucose, lactate and LDH metabolic activity of MOC co-cultures. b) Albumin profiles of liver and skin single-cultures and liver and skin co-cultures in MOC. Data are means ± SEM (n = 6).

turnover was not affected when the daily MOC feeding rate was reduced by half starting on day 7 of culture. Metabolic activity of the MOC co-cultures also showed a biphasic profile with a period of higher but constantly decreasing activity until day 7 and a steady state with low fluctuations both for lactate production and glucose consumption (Fig. 3a). Notably, the steady state phase here coincides with the change of the feeding regimen at the same day.

The glucose metabolism in human hepatocytes *in vivo* is a complex and strictly regulated process. Hepatocyte sources, culture microenvironment and media composition severely alter the glucose metabolism of hepatocytes *in vitro*. Both consumption and release are described for *in vitro* hepatocyte cultures.[14] The liver microtissues used in this study did not show measurable signs of glucose release in any of the experimental settings reported here. We cannot exclude ongoing active glucose release processes in the culture, as they might be hidden due to the immediate consumption by neighbouring HHSteCs. Analyses of the lactate production rate in separate single-tissue MOC-based cultures revealed an average of *ca.* 210 µg per day per circuit for the liver microtissues and 240 µg per day per circuit for skin biopsy cultures at steady state (data not shown). The lactate production profile in co-culture (Fig. 3a) at a steady state (day 8 onwards) had an average value of 350 µg per day per circuit. This slight decrease in lactate production in the co-culture might point to conditional effects between the skin and the liver tissue. The near to steady state consumption of glucose and production of lactate by the co-cultures from day 8 onwards indicates the establishment of a stable artificial coexistence between the two tissues. Preliminary experiments using fluorescence quenching (Presens GmbH, Regensburg, Germany) revealed no oxygen limitation in the circulating media (data not shown). However, inner cells of skin biopsies and liver spheroids might experience reduced oxygen concentrations and might contribute to glycolytic transformation from glucose to lactate.

Albumin synthesis has been selected out of a wide range of functional liver cell activity markers to monitor liver-like activity in the MOC co-cultures (Fig. 3b). The unexpected high amount of albumin in the first 2–3 days of co-culture can be clearly explained by albumin released from the skin biopsies, because the single skin cultures exhibit exactly the same profile. These amounts of albumin are eventually released from the inner lumen of the damaged microvessels of the skin and the extra capillary depots. Strikingly, the albumin profile of the single skin cultures at days 4, 5 and 6 substantiate a pronounced consumption of albumin by the skin. This observation was perfectly matched by the parallel albumin profile of the co-culture at slightly higher absolute amounts. This suggests that the albumin consumed by skin in the co-cultures is provided by the active production of the respective liver compartment. The constant albumin production in single liver tissue culture further backs up this hypothesis. Finally, the crosstalk between liver and skin within the co-culture steadies, and albumin concentration stays close to zero (0.15 µg per day per circuit at day 7 and 0.13 µg per day per circuit at day 14), which we interpret as equilibrium between albumin production by liver and immediate consumption of the same amount of albumin by skin. This matches the well-known facts that albumin is degraded by skin fibroblasts and, furthermore, that nearly 50% of the extravascular albumin in humans is stored in the skin.[15] Albumin is absorbed by the network of collagen, proteoglycans and hyalurons *in vivo*, and its content increases with overhydration in human skin by 20–30%.[15] Therefore, skin biopsies in submerged culture can act as an albumin depot. It remains unclear if skin would be able to absorb even larger amounts of albumin, but this finding again underscores the stable coexistence between the two tissues. It is interesting that albumin synthesis in liver single-organ MOC cultures exhibits values (3.5 µg per day per circuit at day 7 and 6.8 µg per day per circuit at day 14) consistent with those shown in the literature.[14]

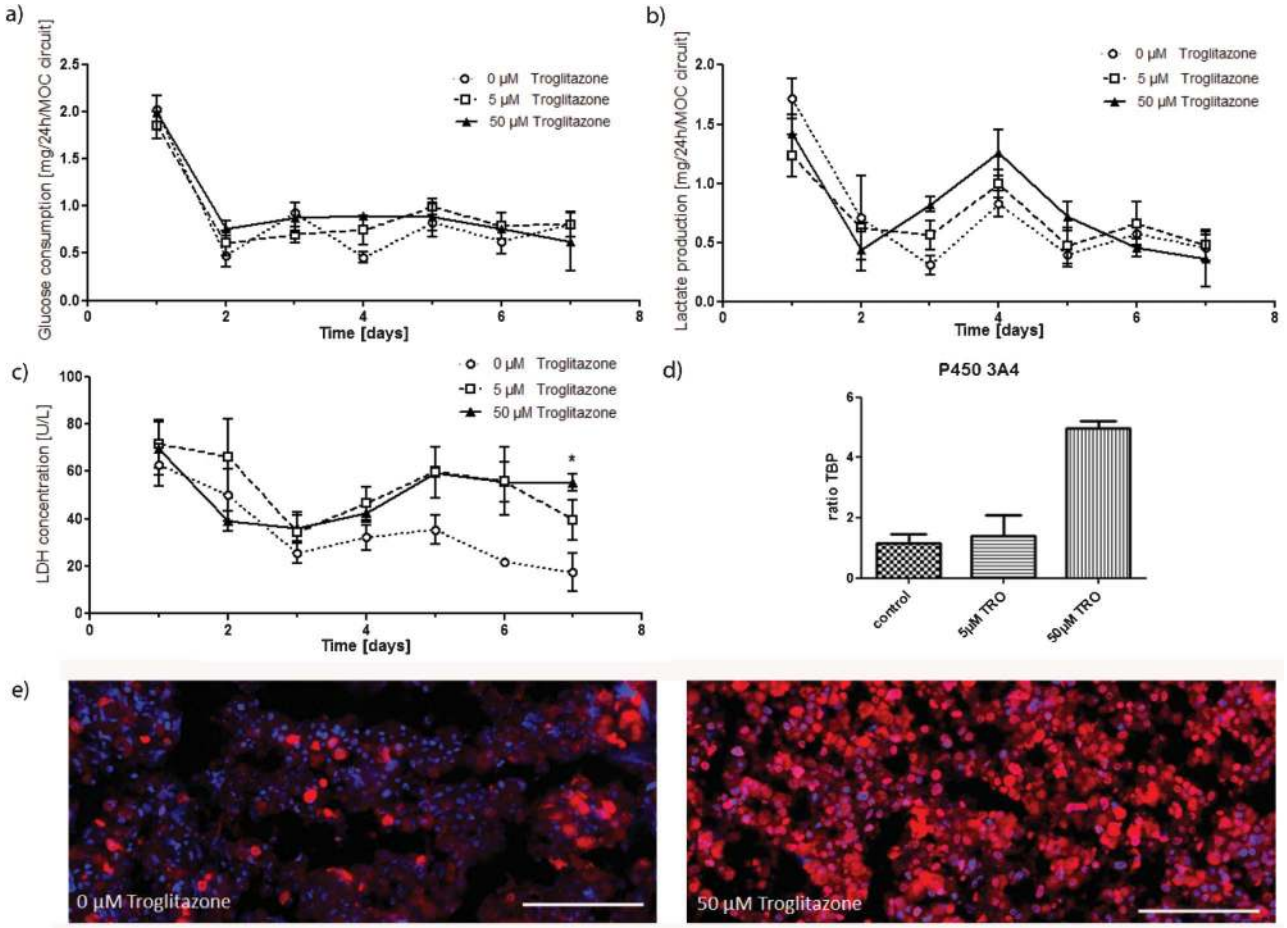

Fig. 4 Sensitivity to troglitazone after 6 days of repeated dose exposure. a) Glucose consumption and b) Lactate production profiles c) LDH values d) Real-time qPCR of the cytochrome P450 3A4 e) Immunostaining of cytochrome P 450 3A4 in control (left) compared to the 50 µM troglitazone exposed group (right), nuclear stain blue. Scale bar 100 µm. c) Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by *post-hoc* Dunnett's pairwise multiple comparison test. $*P < 0.05$ *versus* control. (a, b and c) Data are means ± SEM ($n = 4$).

Multi-tissue sensitivity to troglitazone at fluid flow

Troglitazone was chosen to explore whether the MOC system is suitable for repeated dose substance testing. Its dose-dependent toxicity on HepaRG cells *in vitro* has been described elsewhere.[16] We decided to expose the MOC co-cultures to troglitazone during the first 7 unsettled days of co-culture, because we hypothesised a higher sensitivity to a toxic agent at this stage. Nevertheless, troglitazone had no significant effect on the metabolic activity of liver and skin co-cultures. After 4 days of culture, we observed an increase of LDH activity and after 7 days a significant 60% increase when compared to DMSO-treated controls.

The most sensitive indicator for dose-dependent sensitivity of the MOC co-cultures could be identified on mRNA levels in the respective liver microtissues after cultivation at day 7. qPCR analysis of cytochrome P450 3A4 and immunohistochemistry showed increased expression of this enzyme on mRNA and protein levels in cultures exposed to 50 µM troglitazone (Fig. 4e). This upregulation of biotransformation activity following troglitazone treatment has been shown before[16,17] and indicates a typical reproduction of the toxic mechanism *in vivo*. In contrast to Hewitt *et al.*,[18] we could not detect a pronounced increase of glucose consumption induced by 50 µM troglitazone. The fact that this gene upregulation was not evident on the global metabolic level (in terms of glucose and lactate) might point to a labour division among the HepaRG cells within the microtissues. This hypothesis was disproven by immunohistochemical staining of representative liver microtissue sections after completion of the culture. An evenly distributed overexpression of cytochrome P450 3A4 activity could be detected in all the MOCs exposed to 50 µM troglitazone, whilst only minute expression was detectable in the control group. The experiments revealed a sensitivity of the MOC co-cultures of liver and skin equivalents to a liver toxic substance at different levels of analyses. Long-term analyses extended toward the steady state phase and use of higher doses might give additional insights into the artificial multi-tissue homeostasis in MOCs in further studies.

Performance of Transwell® multi-tissue cultures over 28 days

OECD guidelines for dermal subsystemic repeated dose toxicity testing of chemicals and cosmetics in animals require 21–28 days of exposure (OECD guideline no. 410, ''Repeated

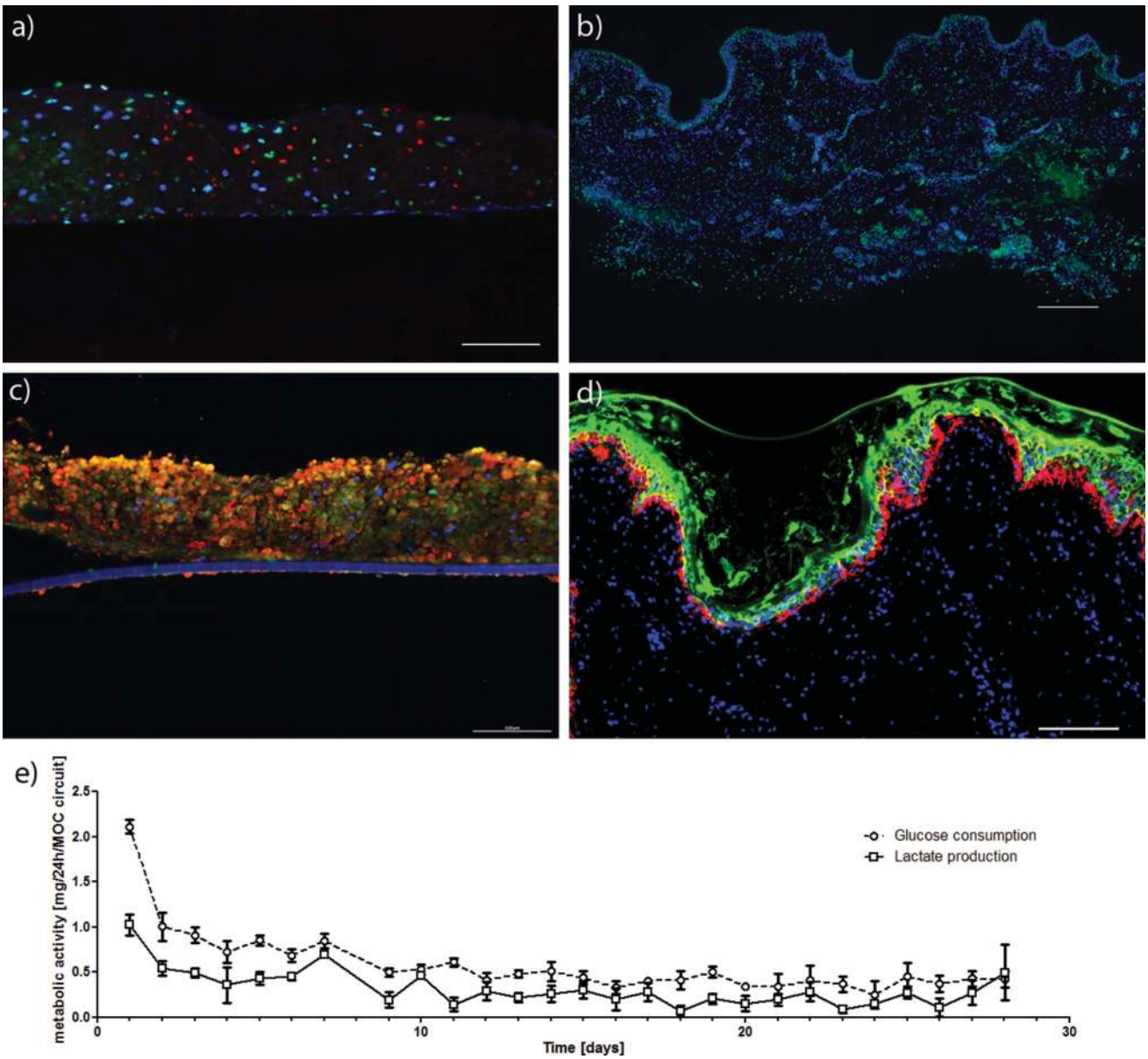

Fig. 5 Performance of Transwell® multi-tissue cultures over 28 days. Evaluation of cell viability by a) TUNEL KI67 staining of liver tissue and b) TUNEL staining of skin tissue after 28-day co-culture in MOC. Cell functionality was shown by immunostaining of c) Phase I enzymes cytochrome P450 3A4 (red) and cytochrome P450 7A1 (green) in liver tissue and d) Cytokeratin 15 (red) and cytokeratin 10 (green) showing both basal and stratified keratinocytes. e) Metabolic activity of the co-culture analysed in culture supernatants. Data are means ± SEM (n = 4). Scale bars: a, b and d = 100 μm; c = 300 μm.

Dose Dermal Toxicity: 21/28-day Study''). We decided to focus on two pivotal aspects to converge our culture system to the current standard animal tests to analyse the long-term performance of MOC-based human liver and skin co-cultures: i) to provide an air–liquid interface for the skin culture for later dermal substance exposure, and ii) to strictly adhere to the required timeline of 28 days of substance exposure. To do so, we decided to use Transwell® inserts to culture liver and skin over this extended period. This would ultimately lead to reduced shear stress and different nutrient supply characteristics in the MOCs. A robust steady state metabolic activity from day 8 onwards throughout the entire culture process could be observed, with the glucose consumption averaging at approximately 400 μg per day per MOC circuit and lactate production averaging at 200 μg per day per MOC circuit. This is about 40% lower than the corresponding values of the 14 day studies and 50% lower than those of the substance-exposed MOC cultures. This might be an indicator of a slightly less efficient nutrient supply through the Transwell® membrane. Nevertheless, the tissues accommodate a perfectly stable long-term performance and, therefore, are very well-suited for relevant substance testing protocols in the near future. Finally, immunohistochemistry of the tissues revealed further insights into their biology: Apoptosis was low in both liver and skin tissues, suggesting an adequate supply of nutrients throughout the whole culture period (Fig. 5a and b).

Liver equivalents were stained for the expression of cytochrome P450 3A4 and cytochrome P450 7A1 and skin biopsies for Cytokeratin 10 and 15 to individually analyse the functions, and thus, the degree of success, of the *in vivo* imitation of each organ. Cytochrome P450 3A4 is an enzyme related to the biotransformation of many xenobiotics, whereas cytochrome P450 7A1 is involved in bile acid synthesis. The expression of both proteins indicates the preservation of a metabolically active phenotype over a culture period of up to 28 days (Fig. 5c). Cytokeratin 15 is expressed in the basal keratinocytes and cytokeratin 10 is expressed in keratinising and non-keratinising stratified epithelia. We could demonstrate that the skin's epidermis still exhibited both basal and stratified keratinocytes after 28 days of culture in the MOC (Fig. 5d); an important factor showing the intact barrier function of the skin. Hence, the MOC system is well-qualified for possible long-term skin penetration and toxicity studies of two-tissue cultures. The cellular metabolism of all tissues was measured for 28 days in the MOC (Fig. 5e). Glucose consumption of the co-cultures varied between different donors, but showed the same trend throughout the culture period. This represents the variety of individual response, which can be crucial for substance testing. Both cell activity and metabolism prove that the MOC system is able to cultivate a combination of two tissues for 28 days with only minor reductions of viability and cell activity. Thus, we were able to demonstrate that our MOC system can be used for culturing various human tissues derived from biopsies, primary cells and cell lines. Furthermore, we were able to culture them in organotypical 3D tissue culture in a fluidic-linked manner, maintaining their viability and characteristic functions for 28 days. Substances could be applied onto the skin, for example, in the form of creams, and penetration can be measured. Furthermore, the combination of skin and liver equivalents in the MOC allows one to analyse the effect of the (cosmetic) substances on the liver, its metabolites and their further effect on the skin. Prepuce biopsies are especially suitable for eye cosmetics due to the similarity of its structure to the eyelid, and similar bacterial flora.[13] In addition, other skin biopsies, for example, from the scalp or the abdomen, and commercially available skin equivalents could be implemented, depending on the appendages and glands needed for application, and substances tested.

Skin biopsies in Transwell® holders achieved better results for skin maintenance than those placed directly in the flow, due to the air–liquid interface of the skin which is only enabled in Transwell® culture and allows the epidermal differentiation in a similar manner to *in vivo*. When the skin biopsies were submerged in the flow, the epidermis was soaked and the shear stress led to premature degradation of the epidermis.

Being able to cultivate biopsies of skin has huge advantages compared with skin equivalents. The biopsies combine all cell types of the human skin, including fibroblasts and keratinocytes, which are usually the only cell types in skin equivalents, and others, such as Langerhans cells, melanocytes, endothelial cells, and the natural extracellular matrix. Due to its originality, the skin can mimic the *in vivo* situation best once its characteristics can be maintained *in vitro*. Being able to prepare and maintain biopsies in a reproducible and robust manner encourages the transfer of other organs into this system. We have achieved this transfer for hair follicles (data not shown).

Exact in-process monitoring of oxygen consumption of the individual tissues will be the next improvement to the MOC platform. This is necessary in order to characterise the energy balance of different tissues in various designs over extended culture periods. The first indicative in-process measurements of oxygen *via* the use of fluorescence quenching and an optical fibre-assisted read-out system (Presense GmbH, Regensburg, Germany) is described elsewhere.[19,20] The oxygen consumption has been explored *via* the easy optical access to each and every position within the MOC. Another future development target, which is currently in progress, is to improve test throughput through process automation.

Conclusion

Co-cultures of human artificial liver microtissues and skin biopsies, each a $^{1}/_{100\,000}$ of the biomass of their original human organ counterparts, have successfully proven the long-term performance of the novel microfluidic multi-organ-chip device. The MOC design presented in this work assisted the co-culture of the two tissues in two culture modes, directly exposed to the fluid flow (proven by 14-day cultures) and shielded from the underlying fluid flow by standard Transwell® inserts (proven by 28-day cultures). The latter mode, in addition, facilitates the exposure of skin to the air-liquid interface. The co-cultures, operated at a total on-chip volume of 600 μl medium at recirculation rates of 40 μl min^{-1} assisted by an on-chip micropump, stabilised at a metabolic steady state within approximately a week. Furthermore, the total fluid-to-tissue ratio in the system supports tissue cross-talk, indicated by the consumption of liver tissue-produced albumin by the skin. Finally, the co-cultures revealed a dose-dependent response to a 6-day treatment with troglitazone at the RNA level. This response was also pronounced when looking at the LDH concentration in the medium and was not detectable at the metabolic activity investigated here.

To summarise, our findings suggest that the MOC platform is capable of comprehensively maintaining various tissues of human origin (cell lines, primary cells and biopsies) over long culture periods. MOC co-cultures can be exposed to pharmaceutical substances at regimens relevant to respective guidelines, currently used for subsystemic substance testing in animals. Furthermore, the platform fits standardised microtissue sizes (liver spheroids) and culture formats (Transwell®) fully implemented in modern industrial substance screening and increasingly accepted by regulatory bodies.

In-depth analysis of the yet undefined fluid flow characteristics of the tissue culture compartments at current device geometries are ongoing to ground a fundamental understanding of the robust long-term stability of the liver and skin tissue co-cultures reported here.

Acknowledgements

The work has been funded by the German Federal Ministry for Education and Research, GO-Bio Grant No. 0315569 and the Russian Ministry for Science, Grant No. 16.522.12.2015 and No. 16.522.11.7057. We thank Kerstin Zenner from the Fraunhofer IWS Dresden for the preparation of Fig. 1b. We thank Dr Guguen-Guillouzo, Dr Gripon and Dr Trepo who made HepaRG cells available.

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