'Human-on-a-chip' Developments: A Translational Cutting-edge Alternative to Systemic Safety Assessment and Efficiency Evaluation of Substances in Laboratory Animals and Man?

Uwe Marx,[1] Heike Walles,[2] Silke Hoffmann,[1] Gerd Lindner,[1] Reyk Horland,[1] Frank Sonntag,[3] Udo Klotzbach,[3] Dmitry Sakharov,[4] Alexander Tonevitsky[5] and Roland Lauster[1]

[1]Technische Universität Berlin, Department of Biotechnology, Berlin, Germany; [2]Universität Würzburg, Würzburg, Germany; [3]Fraunhofer-Institut für Werkstoff- und Strahltechnik IWS, Dresden, Germany; [4]SRC Bioclinicum, Moscow, Russia; [5]Moscow State University, Moscow, Russia

Summary — Various factors, including 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, have generated the need for new solutions to the ever-increasing worldwide dilemma of substance testing. This review provides a historical sketch on the accentuation of this dilemma, and highlights fundamental limitations to the countermeasures taken so far. It describes the potential of recently-introduced microsystems to emulate human organs in 'organ-on-a-chip' devices. Finally, it focuses on an in-depth analysis of the first devices that aimed to mimic human systemic organ interactions in 'human-on-a-chip' systems. Their potential to replace acute systemic toxicity testing in animals, and their inability to provide alternatives to repeated dose long-term testing, are discussed. Inspired by the latest discoveries in human biology, tissue engineering and microsystems technology, this review proposes a paradigm shift to overcome the apparent challenges. A roadmap is outlined to create a new homeostatic level of biology in 'human-on-a-chip' systems in order to, in the long run, replace systemic repeated dose safety evaluation and disease modelling in animals.

Key words: *3-D culture, dynamic bioreactors, microfluidics, microsystems, organ-on-a-chip, toxicity pathway.*

Address for correspondence: *Uwe Marx, Technische Universität Berlin, Department of Biotechnology, Gustav-Meyer-Allee 25, Building 17, Room 580, 13355 Berlin, Germany.*
E-mail: *uwe.marx@tu-berlin.de*

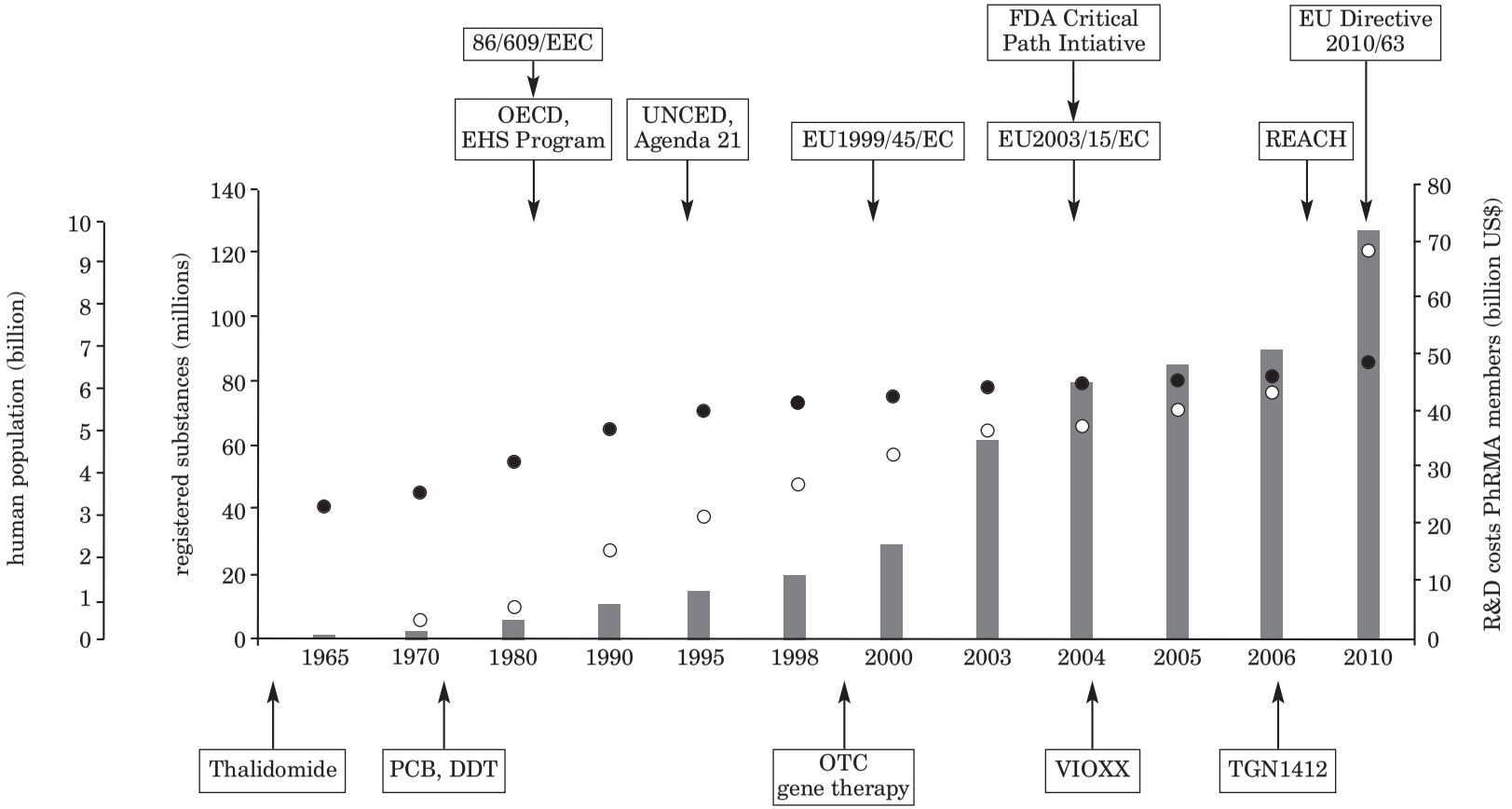
The Global Substance Test Dilemma at the Beginning of the 21st Century

There has quite clearly been an embarrassing gap in the provision of adequate measures to predict the interactions of consumer-relevant natural or synthetic substances with the human body, prior to human exposure, while also taking into account typical environments and individual genotypic specificities. The human population has more than doubled over the last 50 years, whilst the registration of chemicals at the Chemical Abstract Service has increased by two orders of magnitude, from about 212,000 in 1965 to about 88.7 million in 2006 (1), as illustrated in Figure 1.

The variety and potential dosage of chemicals to which each human individual is exposed, have both increased exponentially. The US Environmental Protection Agency (EPA) estimated, based on registrations, a total of 8 million different chemical entities, with a total manufacturing yield of about 12.2 trillion kilogrammes in 2006 (2). Pharmaceuticals, with several thousand approved drugs, represent a small, but, due to direct internal human exposure, very sensitive fraction of these chemicals. In contrast to all other chemicals, they are specifically designed to effectively combat a disease with an acceptable risk-to-benefit balance in patients. According to the United Nations (UN) reporting system (3, 4), more than 624 pharmaceutical products have been banned, withdrawn, severely restricted, or not approved by governments since the thalidomide disaster in the late-1950s. The regulatory bodies have significantly increased their legal frameworks in response to the most devastating misjudgements of the last 50 years (which are summarised in Table 1), and for animal welfare reasons.

The first powerful motivation for revisiting conventional substance testing approaches, i.e. *in vivo* testing, was the scientific concept of the Three Rs (Replacement, Reduction and Refinement), outlined by Russell and Burch (5), which was implemented in the *European Council Directive 86/609/EEC* of 24 November 1986 (6) on the use of experimental animals, and more firmly in the new

Figure 1: Worldwide registrations of chemicals and pharmaceutical research and development (R&D) spending



Population dynamics (●) are shown alongside worldwide registrations of chemicals (○) and pharmaceutical research and development (R&D) spending (bars), reported by the Pharmaceutical Research and Manufacturers of America (PhRMA). The arrows at the bottom of the graph refer to substances related to the most devastating AOPs. The arrows at the top of the graph show the introduction of primary regulations to increase the reliability of safety and efficiency testing.

Table 1: An overview of relevant adverse outcome effects in the last 50 years

Date/case	Adverse outcome effects / Mode of action	Reference
1957–1961 Thalidomide	It was administered as a sleeping pill and an antidote to morning sickness in pregnant women. Thalidomide (or the metabolic breakdown products) inhibits transcription of genes that interfere with normal angiogenesis, resulting in truncation of the limbs.	Fabro *et al*., 1965 (175) Woollam *et al*., 1965 (176) Bauer *et al*., 1998 (177) Stephens *et al*., 2000 (178)
Since 1950s DDT, PCB, Dioxin, etc.	Organochlorine compounds affect the endocrine system by multiple mechanisms. Some act as weak oestrogen agonists, sterilising birds, feminising male embryos, influencing reproductive behaviour, tumours, IQ deficiency and cancer.	Solomon & Schettler, 2000 (179) Bassein, 2004 (180) Harding *et al*., 2006 (181) Patisaul & Adewale, 2009 (182)
1999 Jesse Gelsinger	Ornithine transcarbamylase (OTC) deficiency is a genetic disease that prevents the body from breaking down ammonia, which is a metabolic waste product. Eighteen-year-old Jesse Gelsinger volunteered for a gene therapy experiment designed to test possible treatments for OTC. He died four days after injection of the adenovirus — the vector being used to deliver the OTC gene.	Somia & Verma, 2000 (183) Edwards, 2004 (184)
2004 VIOXX	Specific genetic predisposition is one of the key reasons for the now common and highly publicised drug withdrawal. At the present time, this human genetic diversity is rarely addressed in preclinical and clinical safety studies. A sound hypothesis on the correlation of the morbidity with polymorphic genotypes for 5-LOX and 5-LOX-activating proteins is one of many examples.	Dieppe *et al*., 2004 (185) Arbor, 2004 (186)
2006 TGN1412, 'elephant man'	Super-agonist antibody TGN1412 was developed to direct the immune system to fight cancer cells or reduce arthritis pain. It triggered multiple organ failure in six healthy volunteers who participated in phase I clinical testing. The antibody, by binding to the CD28 receptor, overrides the basic control mechanisms of the whole immune system.	Clair, 2008 (187) Stebbings *et al*., 2012 (188)

Directive 2010/63/EU on the protection of animals used for scientific purposes (7). This animal welfare initiative, based on scientific progress in the field, instigated a marketing ban on cosmetics for which safety testing is still conducted in animals. The 7th amendment to the *EU Cosmetics Directive 2003/ 15/EC* (8), adopted on 27 February 2003, resulted in a marketing ban on cosmetics with animal-tested ingredients in March 2009 (9, 10). The exceptions to this rule were tests for repeated dose toxicity (including skin sensitisation and carcinogenicity), reproductive toxicity and toxicokinetics. A final ban on testing for the remaining endpoints was envisioned for March 2013.

The European regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), adopted on 1 June 2007, will lead to the reassessment of the hazard potential for up to 101,000 chemicals (11). The US FDA's Critical Path Initiative, launched in March 2004, became a driving force for the pharmaceutical industry worldwide, in the search for alternative testing strategies.

Driven by regulatory pressure, an impressive number of scientific initiatives have been funded in the last decade. These are aimed at the transition to a toxicity pathway-based paradigm for chemical safety assessment, with a main focus on the integration of existing *in vivo* data with *in silico* and *in vitro* approaches (12–15). Specific sections of the EU's 6th and 7th Research Framework Programmes aimed to advance the development and validation of Three Rs methods and testing strategies for regulatory purposes. Prime projects in the *in silico* arena have been PREDICT, ChemScreen, Virtual Liver, and eTOX, whilst the improvement of *in vitro* tissue culture tests has been targeted by SEURAT, Sens-it-iv, ACuteTox, ESNATS, carcinoGENOMICS, INVITROHEART, and LIINTOP, among other projects.

Only two human cell culture-based tests have achieved Organisation for Economic Co-operation and Development (OECD) Guideline status so far: the EpiSkin® and EpiDerm® tests. In these, a validated three-dimensional (3-D) reconstructed human primary epidermis layer is exposed to the test substances in a static transwell system for up to 72 hours. In addition, the industry has adopted the eye irritation test EpiOcular® for qualified in-house safety assessments (http://axlr8.eu/work-

shops/2012-landsiedel-endocrine.pdf). Finally, in the area of reproductive toxicology, static embryonic stem cell culture tests are being used to screen substances for embryotoxicity (16).

In the USA, the National Research Council has put forward their *Toxicity Testing in the 21st Century* vision (17), which has been backed by all the relevant governmental institutes (18), to transform environmental health protection (19). The US EPA's ToxCast research programme, with a commitment to transparency and public release of all data, was launched in 2007 (20) with the aim of comprehensively applying batteries of *in vitro* tests to chemicals with known toxicological phenotypes derived from traditional guideline studies on cancer, reproductive impairment and developmental disorders. This programme is currently the most strategic and coordinated public sector effort aimed at transforming toxicology. As of 1 September 2012, 320 chemicals had been analysed in 1057 endpoint assays, of which 312 assays used human static cell cultures. Human cell lines, primarily HepG2 and HEK293, were used in 40% (a total of 125) of the assays, while 60% (187 assays) involved primary human cells. Of the primary human cell assays, 54% (a total of 101) employed single cell types, specifically hepatocytes, HUVEC, dermal fibroblasts, and primary bronchial epithelial cells. The remaining 46% (86 assays) involved combinations of cell types, such as HUVEC + peripheral blood mononuclear cells, HUVEC + vascular smooth muscle, and keratinocytes + dermal fibroblasts. Details are publicly available at http://www.epa.gov/actor. A comprehensive summary on the progress of the aforementioned European and US programmes and projects is publicly available in the annual AXLR8 progress reports for the years 2011 and 2012 (http://axlr8.eu/publications).

In only a few rare cases have static co-culture tests been maintained under industrial conditions. These involve human embryonic or other stem cells (21) that do not require large amounts of oxygen for survival *in vivo*. Therefore, a range of high-quality automation solutions and respective software, such as the Hoffmann la Roche's HDCP plate, have been introduced for high-throughput screening (HTS). The lack of continuous tissue culture homeostasis is the reason why some of these assays have a short testing time.

To summarise, almost all of the regulatory and industry-accepted human cell culture assays rely on static 2-D or 3-D cell culture regimens that provide a short substance exposure time of between a few hours to a few days. The use of primary human cells in testing is increasing, due to their unique human features (22–24), but their use is limited to single exposure hazard assessments. In addition, the assays are limited to the assessment of perturbations in toxicity pathways at the molecular, organelle and cellular levels. Studies on the mode-of-action (MoA) at the organ level, and adverse outcome pathways (AOPs; 25) at the systemic organismal level, cannot yet be implemented. Furthermore, due to the lack of human-like fluid to tissue ratios, it is a challenge to integrate data on artificial *in vitro* substance exposure with dosimetry and human exposure information. Reference dose (RfD), or other exposure guidance values (26–29), in the consumer industry, and quantitative *in vitro–in vivo* extrapolations (QIVIVE) modelling approaches (30–35) in the pharmaceutical industry, offer a growing and promising interpretation of *in vitro* toxicity HTS data. Any move of *in vitro* assays toward a higher degree of biology — organ or even organism level — would eventually permit the transition from hazard-based prioritisation to risk assessment for acute short-term exposure testing.

The Advent of Dynamic Culture Systems

Technical drawbacks of any static cell culture-based HTS assay include the limitation of oxygen and nutrients to a given 3-D tissue size, or monolayer metabolism, and the lack of dynamic protein gradients. Oxygen restriction in cultures of primary human hepatocytes in static culture flasks with a volume of medium higher than 1.2ml, was described in 1968 (36). An initial attempt to solve both problems came about with the invention of hollow-fibre bioreactors in 1972 (37). By optimising the culture conditions, the first functional long-term *in vitro* organ equivalents were developed and introduced into medical practice as extracorporeal bioartificial liver (EBAL) devices (38, 39). These devices were based on a re-assembly of large numbers of primary liver cells in the extra-capillary space of a well-defined hollow-fibre capillary network, which allowed active patient plasma perfusion and supported dynamic protein gradients. In addition, efficient oxygenation was provided to separate capillaries. Death, the AOP for patients with complete liver failure, could be prevented for an extended period until a successful liver transplant was carried out. The same concept — efficient local oxygenation and dynamic protein gradients in the respective microenvironments — was embraced by industry in the development of a human dynamic artificial lymph node model, which aims to predict the human immunogenicity of biopharmaceuticals. This *in vitro* system, in addition to having a physiological oxygen gradient, flushes immune-competent T-lymphocytes and B-lymphocytes through a network of immobilised autologous dendritic cells (40–42). The aforementioned macro-scale systems supported the first medium-term (two to six weeks) organotypic culture systems that were suitable for medical and industrial use. Comprehensive overviews of the

impressive range of macro-scale organ models that exist at research level, but have not yet made it as validated medical and industrial processes, and of all related aspects concerning scientific and ethical constraints with tissue supply, are provided elsewhere (43, 44). The use of such macro-scale systems in the hazard assessment and efficacy testing of substances is compromised by their size and the demand for primary tissue. A feasible option could be the miniaturisation of organ equivalents to the smallest biologically-possible scale.

A Miniaturised 'Organ-on-a-chip' — First Attempt at a Solution

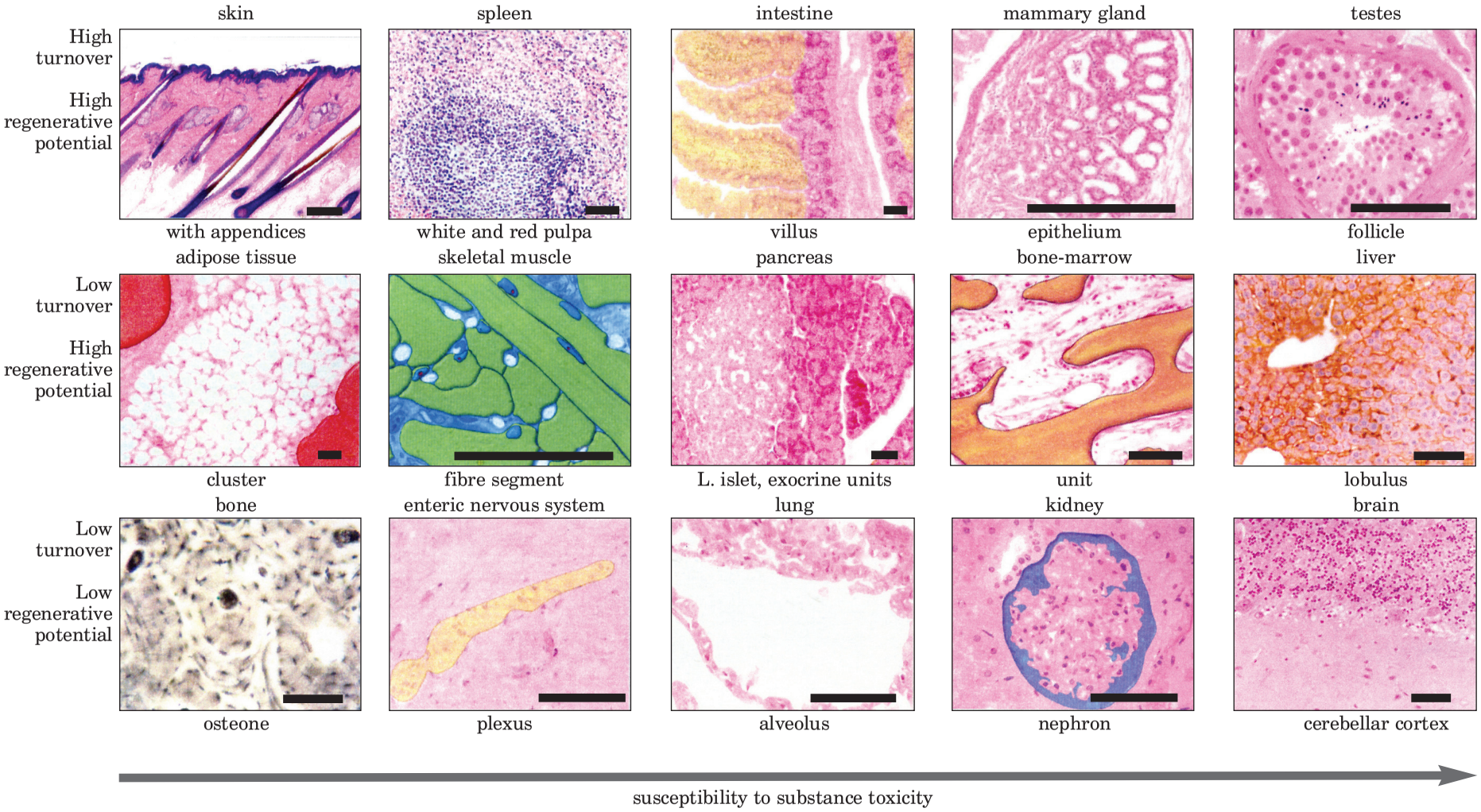
A chance emerged at the beginning of this century, with the use of micro-electro mechanical systems (MEMS), for the development of dynamic micro-scale tissue culture devices, to miniaturise *in vitro* organs to the smallest possible scale. These systems are based on microchannels (usually less than 1mm, and not covered by endothelial cells) for the flow of media, and miniaturised cell culture compartments. Such systems support the replication of shear stresses at physiological intracapillary or interstitial rates, which is mandatory in order to maintain stable protein- and oxygen gradient-based microenvironments. Glass-based and silicone-based devices can be complemented with polymers, textiles, ceramics, or biological matrix entities, and are individually designed to match the well-known requirements of a particular organ microenvironment with regard to shape, surface pattern, stiffness, and micro-architecture (45–47). With the implementation of miniaturised relevant actuators and sensors, MEMS were the first providers in the history of cell culture of all the necessary features for *in vivo*-like tissue-specific electro-mechanico-biochemical signalling. MEMS support expansion and compression forces (especially relevant for lung, bone and cartilage), and microelectrodes for the electrical coupling of muscle tissue (48, 49), or the stimulation of cardiac cells or neurons (50–53). Detailed information on these subjects can be found elsewhere (54–59).

What is the smallest possible degree of organ miniaturisation on such chips? It is important to recognise that a paradigm of stringent correlation between architecture and functionality applies to all levels of biological existence on Earth. These levels of increasing biological complexity have appeared progressively within the multi-million-year process of evolution. These developments were most likely triggered by slight changes in the external environment, which created the conditions for self-assembly to the next level of complexity. Molecules, intracellular organelles, cells, organoids, organs and, finally, the individual organisms themselves, were thought to represent these levels for humans. The role and function of the organoid structures in humans were underestimated for a long time. Today, it is evident that almost all organs are assembled from multiple, identical and functionally self-reliant structural units, which perform the most prominent functions of the particular organ. The multiplication of these structures within a given organ is nature's risk management tool to prevent a total loss of functionality during partial organ damage. With regard to evolution, this concept has allowed organ size and shape to be easily adjusted to the needs of a given species — for example, the liver in mice and men — while following nearly the same arrangement to build up single functional organoids. It is important that these organoids are of very small dimensions, from several cell layers up to a few millimetres. A selection of organoid histologies, all with a prominent functionality and highly variable conglomerate geometry, for the toxicologically most relevant human organs, is shown in Figure 2.

The reactivity of organoids to drugs and biologics is supposed to be representative of the whole organ, because of their independent functionality and a high degree of self-reliance within the respective organ. Therefore, the scale of a single organoid (less than 2µl) is the smallest possible to be engineered on chips. It was recognised that the available micro-shaping technologies were ideally suited to the construction of organoids on minute chips. Therefore, a wave of 'organ-on-a-chip' concepts, covering a wide range of tissues, were proposed during the last decade, although only a small number of devices were actually introduced into research. The chips began with single cell type cultures of various organs, such as endothelial cells (60), myoblasts (61), hepatocytes (62–70), neurons (71), mammary epithelial cells (72), adipose cells (73), and human embryonic cells (74–77). Consequently, in the next phase, heterotypic co-culture systems combining crucial cell types of a specific organoid into artificial functional units were able to more realistically mimic aspects of the lung alveolus (78), the liver lobulus (79–81), the small artery (82), the intestinal villus (83–87), the central nervous system columns (88), and the bone-marrow unit (89). In addition, preliminary studies with combinations of two tissue types were carried out in non-human *in vitro* models — for example, microfluidic devices combining rodent intestine and liver explants (90), and chicken embryonal liver and kidney slices for research, were tested (91). Finally, crosstalk between murine hepatocytes and human umbilical vein endothelial cells was studied in QuasiVivo™ modules (92, 93).

A new concept, to build on-chip biological cardiomicropumps, was recently reviewed (94). After the successful engineering of particular tissues in these individually-designed, research level 'organ-

Figure 2: Alignment of prominent organoids with regard to their susceptibility and physiological cellular turnover



Haematoxylin-eosin staining was used in all cases, except for the skeletal muscle (toluidine blue), liver (tomato lectin) and bone (unstained). The bars represent 100µm, except for the skin and mammary gland, where they represent 1000µm. Some areas in the intestine, adipose tissue, skeletal muscle, bone-marrow, enteric nervous system, and kidney have been artificially coloured to amplify contrast. The primary work-horse of each organoid exhibits an incredibly wide range of lifespans in vivo: *one day (intestinal epithelial cells), 15 days (liver hepatocytes), 20 days (lymphocytes), 30 days (skin keratinocytes), 120 days (blood erythrocytes), 8.5 years (adipose cells), 10 years (bone osteoclasts), 15 years (skeletal muscle cells), 20 years (beta-islet cells), and lifelong (cortical neurons). The images were kindly provided by www.wesapiens.org.*

on-a-chip' systems, some of them were successfully applied to substance testing over a span of several days. These either provided longer test duration (95) or novel toxicity pathway endpoints. A prime example of this was the increased nanoparticle trafficking through the alveolar barrier due to mechanical forces (78) in a chip-based lung equivalent. To our knowledge, none of the current human 'organ-on-a-chip' systems have been used for qualified industrial substance test assays. These might be introduced into industry in the near future, adding significant value to acute toxicity testing for short-term exposures of up to a week at the single organ level. Such single 'organ-on-a-chip' systems lack two major prerequisites to permit systemic safety testing, disease modelling and efficacy evaluation of substances, namely: a) The systemic interconnection of organoids into a human 'body-on-a-chip', which enables physiological interactions; and b) unlimited organismal homeostasis, which ensures tissue renewal and repair or regeneration in the event of pathological damage.

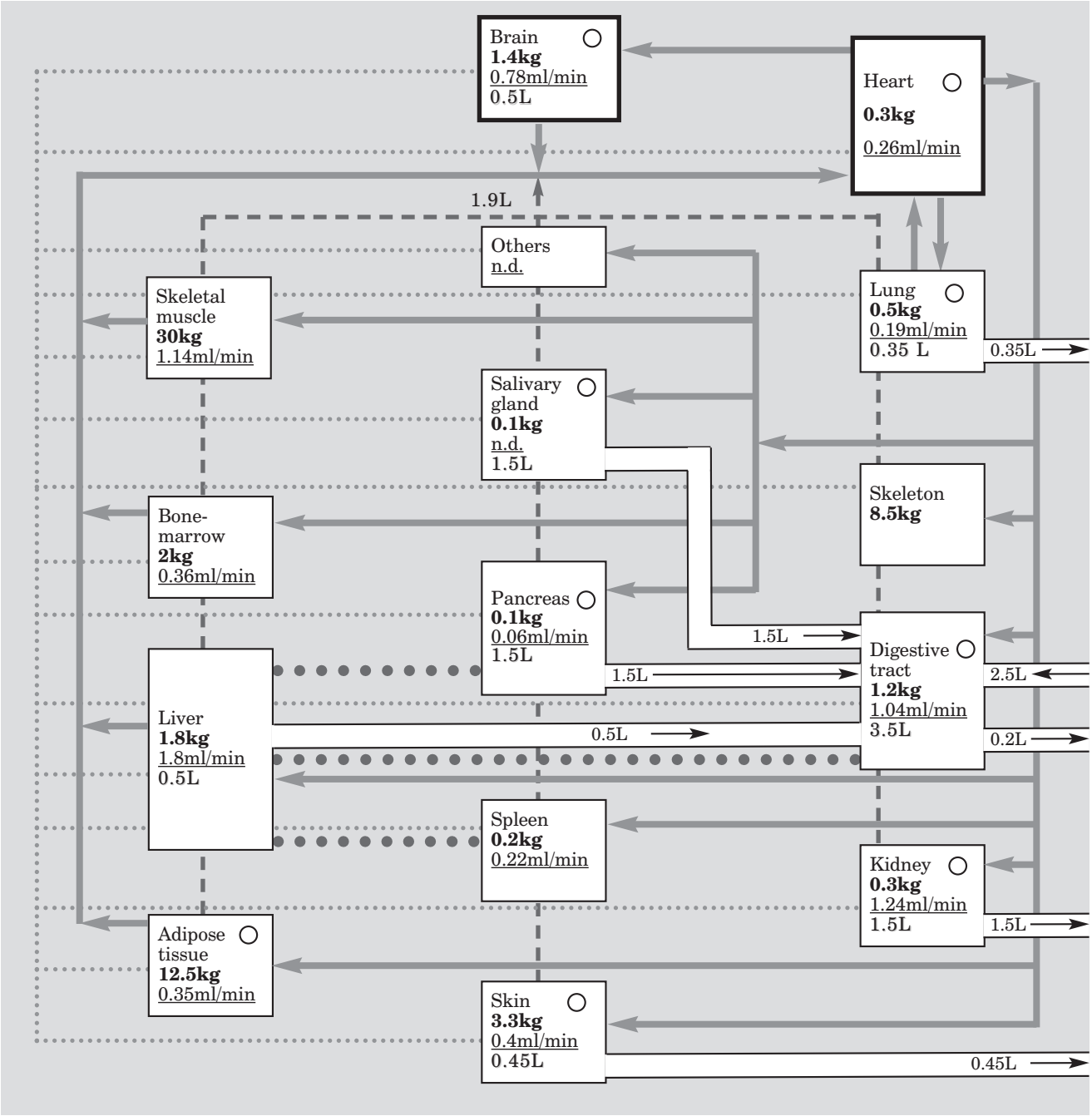
'Human-on-a-chip' Systems — A Feasible Answer

Nowadays, systemic single and repeated dose safety assessments, disease modelling, systemic testing, and efficacy evaluation of substances are carried out on laboratory animals and in humans, due to the lack of predictive alternatives. Relevant international guidelines for chemical testing — OECD Test Guidelines (TGs) 407, 408, 410–413, 419, and 453 — demand a 28-day, 90-day and 12-month test duration, and oral, dermal and inhalation exposure routes in groups of 25–50 animals per substance for safety assessment. The toxicity testing of pharmaceuticals often adheres to approximately the same number and species of animal per candidate drug, and lasts from weeks to months, whilst for safety testing in humans, usually 60–100 healthy volunteers are exposed to a single substance over days or weeks. Notably, the use of animal disease models for the efficacy evaluation of drug candidates has grown rapidly over the last decade. Once an animal model is accepted as a suitable representation of a specific human disease, substance testing is commonly carried out over weeks or months in groups of several hundred animals, similar to human patients in clinical phase 2 trials. A translational alternative to these tests and trials should ideally narrow down the phylogenetic distance between laboratory animals and human beings, and close the biosimilarity gap between the current human 'organs-on-a-chip' and human beings. The definition of spatio-temporal biological levels is of outstanding importance for 'human-on-a-chip' concepts, due to the biological fidelity of an individual during its lifespan. It is evident in the substance testing and disease modelling arenas that prenatal development, childhood and adulthood, at the gender level, are discrete phases of human biology in an individual's lifespan. Considering the ever-increasing human lifespan, senescence is envisaged as a new category. In addition, the period of pregnancy is also a category to be considered (96). The majority of on-chip developments focus on the imitation of non-pregnant adulthood, as this time span has the largest numeric relevance for the safety and efficacy testing of substances. Exceptions to this are the field of reproductive toxicity (16) and the human 'embryo-on-a-chip' developments. A concept called 'body-on-a-chip' (if non-human cells are involved) or 'human-on-a-chip', albeit at a very rudimentary level, could be the answer to the dilemma of systemic testing. With the introduction of such a conceptual target, another two consecutive levels of biology — the systemic arrangement of organs by using technical channels for organ interconnections, and the long-term homeostasis by using endothelial cell-based vasculature for organ interconnection — have become the real challenges for tissue engineers.

The first level represents the short-term maintenance of different human cells, or tissues, arranged systemically on a chip. The systemic arrangement depends on the development and application of feasible microfluidic multi-tissue devices, which need to support heterotypic tissue survival for days or even weeks. The second level constitutes the self-reliant long-term homeostasis of groups of organoids at a miniaturised organismal level. These groups need to rely on an evolutionarily optimised specialisation of each organ toward its primary function at a minute organoid level, against the background of strict interdependence of all organs within an individual for survival. Important physiological parameters for the maintenance of such homeostasis are shown in Figure 3. A typical 35-year-old, 73kg male is used to qualify the design criteria for any 'human-on-a-chip' concepts.

Any systemic *in vitro* model needs to ultimately emulate human organ and system functions as closely as possible within their self-reliant interplay. Macro-scale bioreactor systems, providing systemic arrangements of *in vitro* organ models for tissue culture, were proposed by Marx and co-workers from the department of Clinical Immunology of the Charité Medical School, Berlin, back in the 1990s, and were based on a systemic arrangement of hollow-fibre cartridges (EP0584170B1: *Process and device for cultivating different mammal cells at the same time*). At the same time, Sweeney and co-workers from the Department of Pharmacology at Cornell University, Ithaca, New York (97), created an analogous system that was based on an arrangement of

Figure 3: Human organismal homeostasis at rest



kg = *organ weight;* ml/min = *organ blood flow;* litres = *daily fluid secretion rate.*
→ = *venous flow;* ····· = *nervous system;* – – – = *lymphatic flow;* ● ● ● ● ● = *hepatic system.*

A 35-year-old, 73kg man, at rest, needs 430 litres of oxygen and 1800kcal energy per day — 2.5 litres of nutrition are needed to supply this energy. This amount is associated with exactly the same volume of fluid that is excreted via urine, lung, skin and faeces. It is important to note that the brain and muscle (cardiac, skeletal and smooth) rely on electrical coupling. Mechanical forces are present in each organ, ranging between high for the skeleton, to low for the bone-marrow (blood flow shear stress only). The man is 60% liquid (43.8 litres); one third of this is extracellular (14.6 litres). The latter is processed and recycled daily through and between the organs. A 5L/min blood flow is pumped through this organism to ensure fluid dynamics, and provide oxygen and immune defence. The pattern of shear stress transmitted by this blood flow is quite different on the organoid level, and depends on the capillary type (discontinuous, fenestrated, closed) and on the micro-architecture of the organoid. Organs which have a secretion, or excretion are marked ○.

glass culture vessels. Only the appearance of MEMS technologies brought these historical approaches into a recent tangible reality. A miniaturised and newly designed version of the latter system was proposed in 2002 by the same group (US7288405B2: *Devices and methods for pharmacokinetic-based cell culture system*). This system was called a micro cell culture analogue (μCCA), and was brought into play for pharmacokinetic and pharmacodynamic (PKPD) modelling by Shuler's group in 2004 (98). The layout of the μCCA has progressed over the last few years (99). To our knowledge, it represents the first and, until recently, the only dynamic micro-scale system supporting physiologically-based pharmacokinetic, quantitative structure–activity relationship (QSAR) and QIVIVE modelling (recently reviewed; 100). It combines cultures of different human tissues into a common media flow for the prediction of the time-dependent concentration of a parental compound and its metabolites. The principal design of the system is schematically illustrated in Figure 4. It relies on the recirculation of a common culture medium, with around 10,000 cells per culture compartment, and permits substance exposure times of up to four days.

The proof-of-concept was provided in studies with naphthalene as a model toxicant (101). Furthermore, two combination therapies for improved Doxorubicin (102) and Tegafur treatment (103) have been tested in human cancer cell lines in μCCA layouts, which were specifically modified for those applications. The system, at its selected scale, provides *in vivo*-like tissue mass ratios inside the culture compartments, a culture medium inflow split that is equivalent to the respective blood flow split in humans, and relevant residence times in the tissue compartments. Furthermore, the microfluidic chip design is claimed to support physiological shear stress and liquid–cell ratios comparable to those of the respective organs, and it can be operated over periods of up to four days. However, in order to advance the μCCA technology into a routine test assay platform, the following issues remain (100):

— the avoidance of pump systems by designing a 'pumpless' chip with a gravity-based culture medium flow;

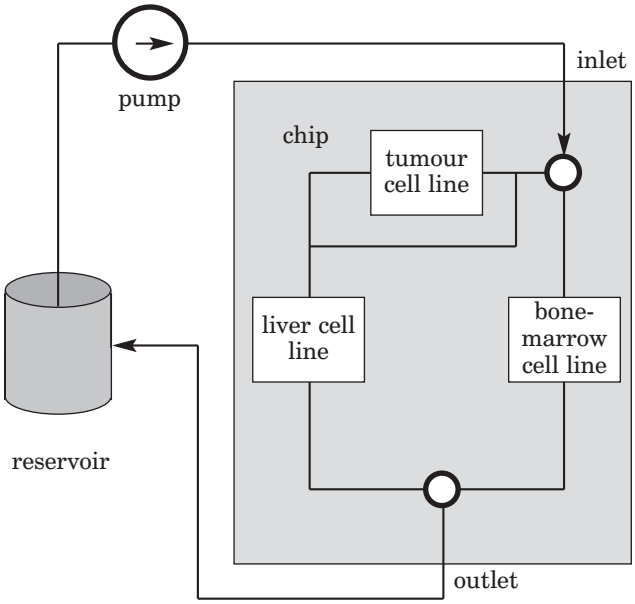
— improvement of the monitoring and control of the periphery (in-process controls for viability and metabolic activity, e.g. pO_2 sensors);

— the maintenance of mechanical forces, or electrical coupling, where relevant;

— the optimisation of the standard culture medium into a more realistic blood surrogate; and

— the upgrade of the organotypic characteristics of cell culture compartments from 3-D human cell line constructs toward primary human organoids, addressing their specific extracellular matrix architecture, epithelial barriers, stromal tissue impact, physiological absorption, and secretion or evaporation kinetics of water, where relevant.

Figure 4: A scheme of the flow diagram of a μCCA



The chip is 25mm by 25mm and the flow channels are 20–100μm wide. The flow is laminar and, typically, more than 10,000 cells populate each tissue culture compartment. The design is based on the Hagen-Poiseuille Law, which allows human-like fluid velocity to be matched in each channel, and liquid residence time in each compartment to be equivalent to that in the respective PKPD model in silico.

Research into various aspects of these challenges has recently generated some progress (104–107).

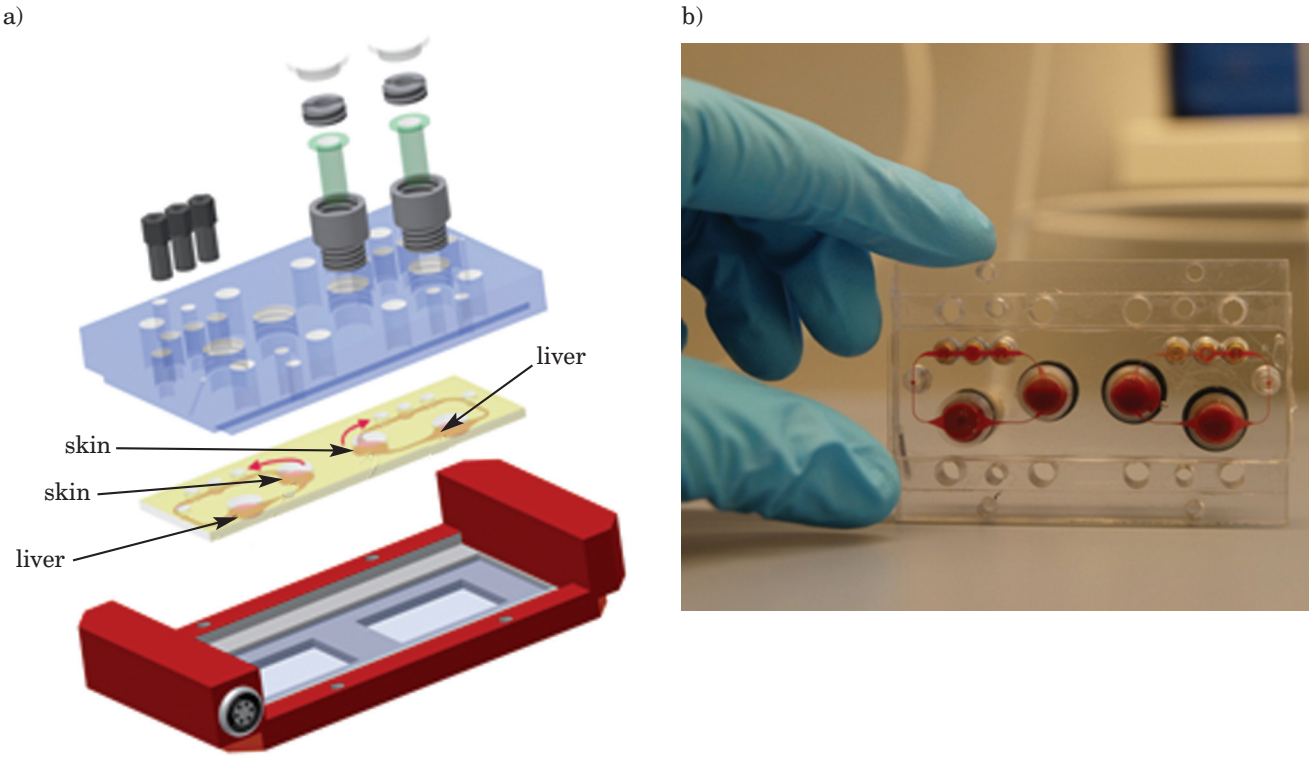
Three other microfluidic systems, targeting systemic arrangements of different human tissues on a chip, have recently emerged. Zhang and co-workers from the Institute of Bioengineering and Nanotechnology, Singapore, have developed a multi-channel 3-D microfluidic cell culture system (μFCCS) with four separate channel-based cell culture spaces that can each be loaded with tens of thousands of cells. Cell line-derived human liver, lung and kidney cells, and primary human adipose cells, are shielded from direct shear stress in the respective channels. The main means of transport of nutrients and test substances between cell and perfusion compartments is through diffusion. Recirculation at flow rates of 0.2ml/min, and a two-day exposure to tumour growth factor β1, led to a discrete biological response in the human lung

cells, whilst the other compartments remained uncompromised (108).

Another integrated systemic model for the emulation of intestinal absorption and hepatic metabolism, and for the assessment of the responsiveness of human breast carcinoma cells to four commonly used drugs, has been reported by Imura and co-workers from the Department of Applied Biological Chemistry, School of Agricultural and Life Sciences of the University of Tokyo. Their system provides a unidirectional flow in a chip format, the area of a microscope slide. It supports the constant perfusion of tens of thousands of human HepG2 cell line-based hepatocytes and human MCF-7 cell line-based human breast carcinoma cells, consecutively arranged in a single microchannel. Drugs are provided through a tightly closed monolayer of human CaCo2 cell line-based intestinal epithelial cells into the culture medium flow through the channel before the liver compartment, which reproduces the absorptive properties of the human intestines. Exposure was achieved for two days at a flow rate of 0.4µl/min, and led to discrete biological effects on the human breast carcinoma cells (109).

Finally, a pumpless system has been designed in the same standardised chip format (76mm × 26mm) that was prototyped by Marx and co-workers at the Institute of Biotechnology at the Technische Universität Berlin. This design should support the simultaneous maintenance of explant cultures from human liver, bone-marrow and brain tissue, and a stem cell niche in a unidirectional gravity-based liquid flow (110). It has recently been redesigned and equipped with a robust peristaltic on-chip micropump, modified from Wu *et al.* (111). The resulting platform, called a multi-organ-chip (MOC), supports either unidirectional flow perfusion or the recirculation of culture medium through one or more tissue culture compartments (Figure 5). The tissue culture compartments support the integration of matrices for organ model-

Figure 5: A multi-organ-chip platform



a) A PDMS-chip (yellow), 3mm thick, bonded onto a microscopic slide (26mm × 76mm), hosts two independent microcircuits with a circulation channel of 100µm × 500µm. Each channel connects two tissue culture compartments (6mm). The tissue culture compartments support the integration of matrices for organ modelling, e.g. the liver, and the integration of standard 96-well inserts (green) with 10µm-thick microporous polyester membranes for biological barrier modelling, e.g. the intestine or skin (93, 94). The micropump (in black) consists of three consecutive elastic membranes over the channel, actuated at a specific sequence for pulsatile unidirectional flow and an adjustable frequency for the flow volume, flow rates and residence time in organoids. b) Represents a worm's-eye view of two blood-perfused circuits.

ling, such as the liver, and the integration of standard 96-well inserts with 10µm-thick microporous polyester membranes for biological barrier modelling, e.g. the intestine or skin (112, 113). Rapid prototyping tools allow microchips to be easily adjusted to any desired format, in the area available, within very short time-frames. Preliminary tissue culture experience has been gained with this platform by culturing human liver and skin equivalents in a consecutive cyclic arrangement over 28 days (http://axlr8.eu/workshops/2012-marx.pdf).

An industrially-validated high-throughput test assay platform for systemic single dose testing and short-term exposure safety assessment might evolve from any of the ongoing 'human-on-a-chip' engineering approaches. The application of reversed dosimetrics with QSAR-estimated partitioning coefficients and metabolism parameters (114, 115), will allow human-relevant data to be derived from test assays based on such 'human-on-a-chip' systems. This will reduce, or even replace, acute systemic toxicity testing in animals. Optimisation toward standardised cost-effective automated handling is the prime focus in order to finally complete this first level of systemic human biology *in vitro*. However, the existing 'human-on-a-chip' or single 'organ-on-a-chip' solutions differ significantly from human biology in two pivotal aspects: the inability to provide unlimited organoid homeostasis, and the lack of physiological regenerative capabilities of cellular repair. Therefore, for long-term exposure testing, it will be necessary to climb to the next level of systemic biology — long-term homeostasis of primary organoids at a miniaturised organismal level *in vitro*. We hypothesise that the lack of a dynamic interplay between organ-specific cell types, with their vascular and stromal tissue bed, and the absence of adult stem cell and progenitor niches for local regeneration, are responsible for the crucial missing capabilities of current 'human-on-a-chip' systems.

Vasculature, Stroma and Adult Stem Cell Niches — a Paradigm Shift in the Puzzle

The imitation of the four systemic components of a human body — the vasculature, stromal tissue (also termed mesenchyme) and nervous and immune systems — on a chip is a unique and outstanding chance of improving the existing 'body-on-a-chip' systems. The cardiovascular and nervous systems are the nutrition supplier and regulator, respectively, in the harmonised concert of organs in our body (see Figure 3). Consequently, their lack of function almost immediately leads to death, and is represented in medicine by a flat line in an ECG and EEG. The morphogenesis of the two systems is heavily linked (116, 117), and continuous neurovascular crosstalk is an important factor of the physiological behaviour of the nervous system (118). The importance of the blood vasculature extends far beyond the nervous system, as it supplies nutrients and hormones (plasma), oxygen (erythrocytes), immune defence (leukocytes), and coagulation capability (thrombocytes) to each and every organoid in the body.

The plasticity of approximately 60 trillion endothelial cells (ECs) creates an unmatched structural heterogeneity in response to the local environments of the different organoids in the body, under various shear stress conditions, and with diverse morphological and molecular signatures. Continuous types of capillaries (for example, in the heart, lung, kidney and brain) significantly differ in pore size and sieve functionality from fenestrated ones, which are found in the endocrine organs and in the glomeruli of the kidney, and discontinuous types of capillaries present in the liver, bone-marrow and spleen. This phenotypic heterogeneity of ECs is likely to provide at least two fitness advantages: a) It allows the endothelium to conform to the diverse needs of the underlying tissues throughout the body; and b) it provides the endothelium with the capability to adapt to different microenvironments, e.g. the profoundly hypoxic and hyperosmotic environment of the inner medulla of the kidney *versus* the well-oxygenated environment of the pulmonary alveoli (for comprehensive reviews, see 119–123). Another advantage is the fact that the venous endothelium builds a blind-ended network that is connected to lymphatic vessels for returning about 1.9 litres of lymph fluid per day into the blood circulation (124), as shown in Figure 3. This particularity makes human primary microcapillary ECs the best target for connecting multi-organ arrangements *in vitro*.

An equally important aspect of human vasculature is the capability of generating and repairing tissues through angiogenesis (125–131). These factors have encouraged experiments on the establishment of *in vitro* vasculature, both for research at a microfluidic scale (60, 82, 132) and to engineer whole organs for transplants. The latter research has revealed that EC crosstalk with the respective cell types within the organoids and with the stromal bed, regulates local and global cell-specific and organ-specific branching patterns during developmental and remodelling processes. The stroma also regulates epithelial differentiation and branching itself, and plays an essential role in the formation and maintenance of adult stem cell niches crucial for organ homeostasis and regeneration in humans (for comprehensive reviews, see 133–140).

There have been tremendous efforts to develop macro-scale bioreactors for the creation of vascularised human organ equivalents for transplants,

but their success has been limited (for reviews, see 140, 141). The high remodelling capacity of the stromal and vascular components of an autologous vascularised tracheal implant manufactured in a dynamic macro-scale bioreactor has been demonstrated by Walles and co-workers, from the Department of Cell and Tissue Engineering of the Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart (142, 143). To achieve this, de-cellularised vertebrate jejunum (144) was stably re-populated with a human endothelial vascular network in a pulsatile flow, and human stromal fibroblasts and smooth muscle cells were seeded into the collagen matrix of that scaffold. A similar *in vitro* pre-vascularisation approach supports the *de novo* formation of human bone tissue after damage (145).

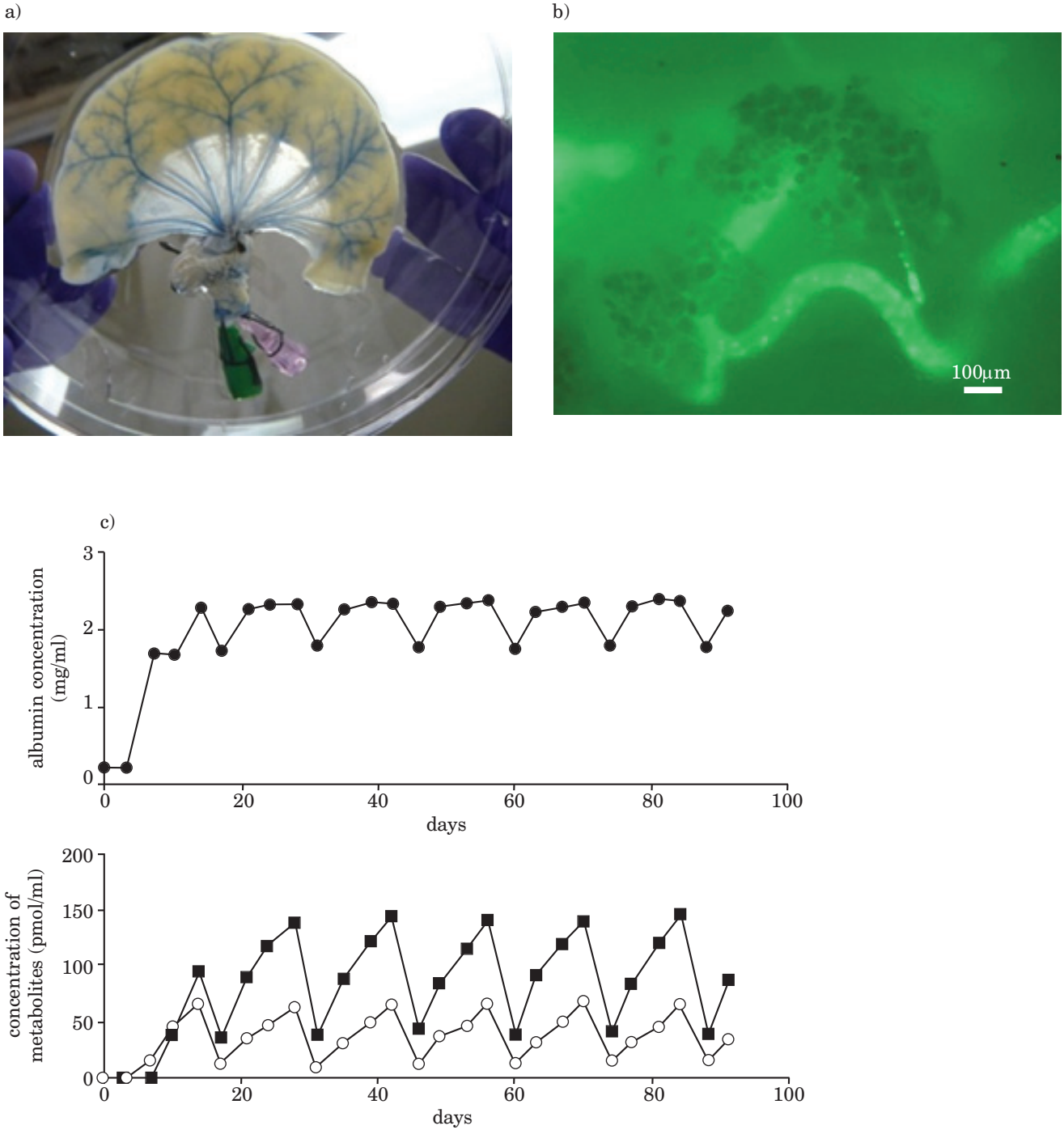
The question that arises is whether such a biologically-inspired approach would work in humans, should the technology be able to provide a macro-scale solution for the long-term homeostasis of human organ equivalents *in vitro*. This might ultimately overcome the reported short-term functionality of all existing *in vitro* test systems, including microfluidic ones. Indeed, as shown in Figure 6, the group has successfully adapted the macro-scale bioreactor technology and the BioVaSc® system to *in vitro* testing demands (146), and has shown the unlimited metabolic functionality of human liver equivalents. The experiments were stopped after three months, at stable functionality, in order to perform immuno-histological investigations. The extent to which the system supports long-term substance exposure studies remains to be discovered. These early discoveries underpin the principal tool for the creation of unlimited organ homeostasis in macro-scale single liver equivalents *in vitro*, by providing a vascular and stromal bed within a dynamic shear stress environment. The limitations of macro-scale systems for substance testing were discussed earlier in this review. Therefore, a transfer of these principal solutions for *in vitro* vascularisation into microfluidic 'human-on-a-chip' platforms is essential. Preliminary data recently derived at the Technische Universität Berlin revealed that the establishment of a microcirculatory vasculature in chips of the MOC platform, over at least 14 days, is feasible at a continuous pulsatile flow of medium (see Figure 7).

The branching of the vasculature into smaller vessels and capillaries remains a prime challenge to the optimisation of the organoid culture compartments of the MOC platform. An elegant solution to branch arteriola-sized vessels into smaller ones has been recently provided by printing rigid 3-D filament networks of carbohydrate glass as sacrificial templates (147). This technology might be instrumental to overcoming this challenge. In addition, the vast know-how gained from artificial artery implant engineering might be useful to increase chip-based arterial vessel size (for a review, see 148).

Another important factor in human organogenesis is the ability to spontaneously self-assemble tissue compositions into minute tissue architectures by, for example, mesenchymal condensation prior to vascularisation and angiogenesis (149). Such human morphogenic processes could eventually be mirrored, even in static oxygen-limited cultures. Prime examples of this are the human epidermis equivalents, human hair microfollicles (150), human neuronal tissues (151) and the intestinal tissues described earlier (152, 153). The authors hypothesise that, *in vivo*, the formation and growth of such structures toward differentiated agglomerate precursors is crucial to promote angiogenic sprouting from the nearest blood vessels. Subsequently, this also provokes the final formation of, for example, a liver lobulus, a terminal hair follicle or a lung alveolus through vascularisation *in vivo*. These mechanisms *in vivo* always need the presence of stem or precursor cells interacting with a proper stromal environment. Therefore, it is not surprising that almost all human adult organs contain stem cell niches with distinct organ-specific anatomy and function (154–156), which are capable of regenerating respective organoids. Examples of human stem cell niches include: a hepatic oval stem cell niche in the liver (157, 158), a follicular bulge stem cell niche in the skin (159), a crypt base, columnar stem cell niche in the small intestine (160), a broncho-alveolar stem cell niche in the lung (161), a haematopoetic stem cell niche for blood reconstitution (162–164), a sub-ventricular zone stem cell niche for the regeneration of nerve tissue (165), a satellite stem cell niche in skeletal muscle (166), and a cardiac stem cell niche (167). Stable stem cell niches in organotypic static skin cultures supported unchanged tissue functionality over three to four weeks (167).

This interplay, as described, is particularly prominent *in vitro* when culturing organoids derived from rapidly proliferating and high turnover organs with epithelial barriers, such as the skin or intestine (Figure 2). Here, the remaining crucial factor facilitating the flexibility of human biology, called mesenchymal–epithelial transition (MET) and epithelial–mesenchymal transition (EMT), comes into play (for reviews, see 168–171). These types of transition between fibroblasts and epithelia have been evolutionarily conserved for two reasons: a) For a proper organogenesis in prenatal development; and b) for the ultrafast repair of wounds, as it is essential that vertebrate carnivores recover quickly for further hunting. These evolutionary mechanisms point toward a tremendous plasticity of mesenchymal precursor cells, stromal (mesenchymal) fibroblasts

Figure 6: Long-term homeostasis and functionality of vascularised macro-scale liver tissue cultures

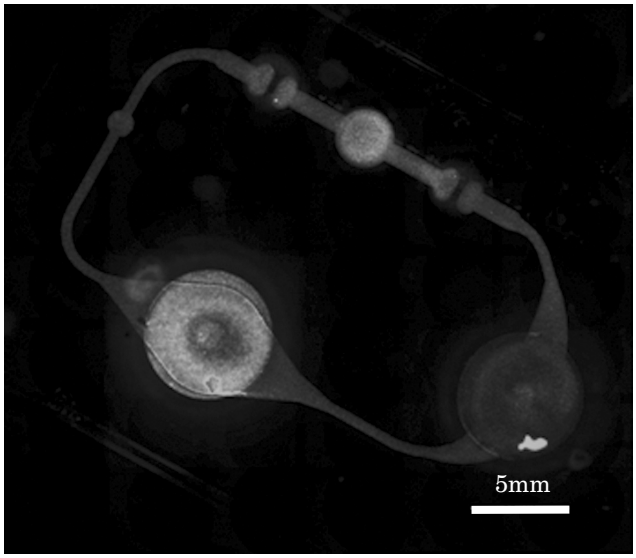


a) Viable human vasculature (stained blue) was restored in BioVaSc® matrices. b) Primary human liver tissue cultures of 5–8cm³ in size were established on BioVaSc® matrices (in triplicate). Fluorescence staining of the endothelial cells (stained green; scale bar = 100µm) shows the vessels surrounded by the unstained dark liver cell clusters. Vessel sprouting into the tissue can be hypothesised for the microvessel on the image. Constant circulation of culture medium from a 0.5-litre medium reservoir was used for nutrition. The medium was exchanged every five days. The liver equivalents were exposed to dextomethorphan, a cough suppressant know to be metabolised in the human liver through Phase II metabolism into dextrorphan-glucuronidide. c) The synthesis of albumin indicates homeostasis and correlates to the stable metabolism during the entire experiment.

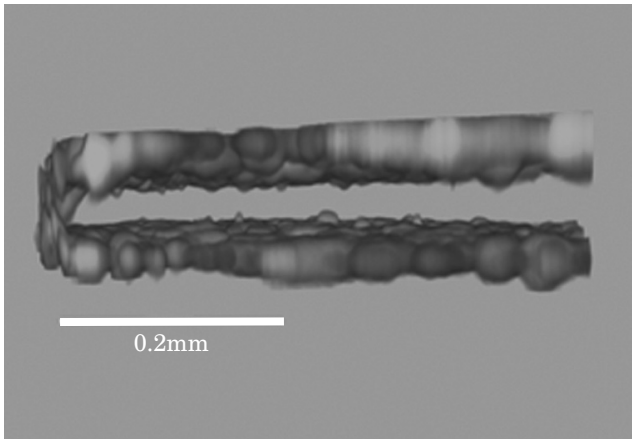
○ = *dextrorphan-glucuronidide;* ■ = *dextomethorphan.*

Figure 7: The endothelialisation of MOC microsystems for the provision of vascular beds

a)



b)



a) 1.2 × 10^5 GFP-transduced cells of the human microvascular endothelial cell line HMEC-1 were cultured over 14 days in each of 10µl microfluidic flow circuits of the MOC platform with a 100µm × 500µm (H × W) channel geometry. The circuits were constantly perfused in a circulating mode at flow rates of 40µl/min. Endothelial cells covered the entire surface of the channels and the tissue compartments, as verified by their vital green fluorescence imaging in the circuit seen as lighter grey (bar = 5mm). b) Two-photon imaging microscopy confirmed the complete and tight closure of the channels, including the side walls. An image of the upper, lower and left side walls of a channel section demonstrates the full endothelial cell coverage (bar = 200µm).

or epithelial cells, that allows them to flexibly form specific cellular microenvironments inside a specific organ. The authors finally hypothesise that combining organoid-specific vascular and stromal beds, with respective adult stem, or progenitor, cell niches in microsystems that, where relevant, provide proper mechanico-electrical coupling (45), can lead to unlimited homeostasis and natural regenerative capabilities in 'human-on-a-chip' systems. *In vivo*-like colonisation and sanitisation of organoids from physiologically occurring cell debris or pathogens is envisioned, if whole blood circulation is to be applied.

A Roadmap Toward the Ultimate Level of Human Biology on a Chip

In addition to the consequent transition of current 'human-on-a-chip' approaches into valid alternatives for acute systemic toxicity testing, we propose a paradigm shift for the establishment of unlimited on-chip organismal homeostasis with natural regenerative capabilities *in vitro*.

Human endothelial cell-based vasculature should, therefore, form and interconnect several microvascular beds into a common blood circulation. Each of the micro-vascular beds needs to be integrated into an organ-specific stromal tissue bed, which provides the respective extracellular cell matrix (ECM)-based micro-architecture for proper organoid assembly. Stem cell niches should be implemented. At least three different options can be explored to create organoids on such chips:

— self-assembly by adding respectively differentiated cell types followed by neo-angiogenesis;

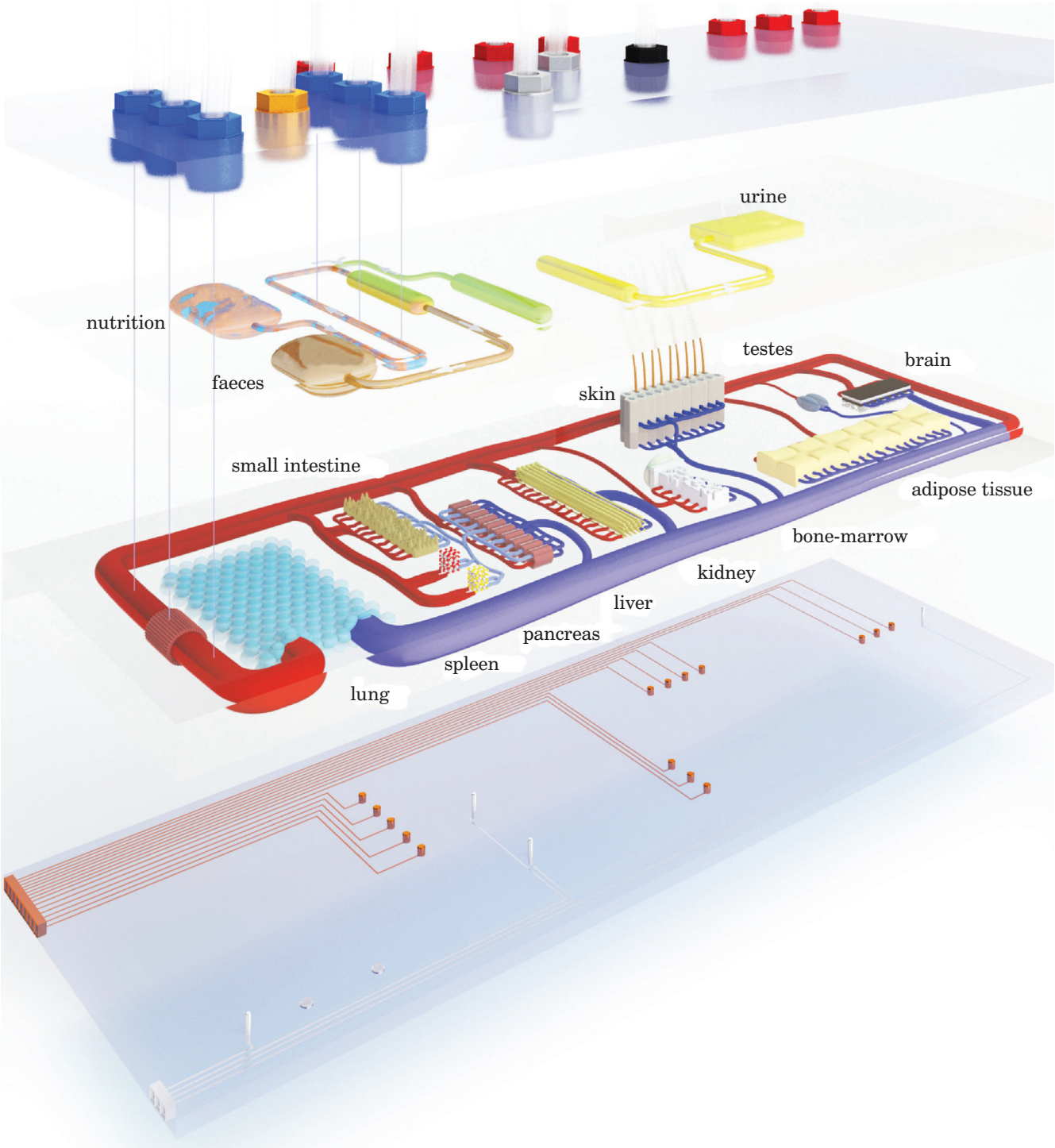
— *de novo* formation from adult stem cell pools along with neo-angiogenesis; and

— connecting tissue explants containing groups of organoids into the pre-established vasculature.

In all of these cases, finely orchestrated organ-specific groups of fully functional organoids should maintain their specific functions in concert. Figure 8 shows an example of a 'human-on-a-chip' concept developed to fit the MOC platform format.

The radical paradigm shift proposed here is a desperate challenge. Among others, it demands the provision of actuators and sensors to the 'human-on-a-chip' platforms that match the functions of their *in vivo* counterparts. Actuators should couple a broad range of mechanical stresses differentially into relevant organs at natural degrees. Sensors with exceptionally high sensitivity should be devel-

Figure 8: 'Man-on-a-chip' at 0.00001 scale



Here is a possible design for maintaining eleven human organ equivalents in a common blood vasculature on a chip of the MOC platform. The microfluidic device consists of three layers. Organ equivalents are arranged in a middle PDMS layer. The liver equivalent is designed to hold ten liver lobuli. All the other organ compartments are designed to hold a number of their organoids that is proportional to the ten liver lobuli. The upper PDMS layer holds the antra for nutrition and bile provision to the intestine. It also supports urine and faeces removal, which are designed with a two-week exchange capacity. The lower glass-based sensor layer comprises sensors for tissue 'innervation' and the measurement of pO_2, pH and temperature, and proper barrier functions. A separate holder on top of the chip fixes 17 air pressure-based actuators that mimic heartbeat (blue), intestinal peristaltic movement (blue), lung air-flow (brown), bone compression (black), arteriolar constriction (red) and urine and bile well development (grey).

oped to cope with minute sample volumes for the main parameters of human organismal homeostasis, such as: organ viability, tissue temperature, pH, daily fluid balance, intracapillary pressure, blood flow volume, oxygen and nutrient consumption, fluid absorption and intestinal juice secretion, albumin and bile synthesis, urea excretion, ion balances, osmolarity, and electrical coupling. Miniature organ sizes and contact-free access to the transparent bottom of the chips might allow the use of strong in-process research tools, such as, two-photon microscopy for tissue imaging, fluorescence ratio imaging for local interstitial pH measurement (172), phosphorescence quenching microscopy for interstitial pO_2, or infrared spectroscopy to detect physiological stresses (173). Systems biology approaches for the identification of physiological performance, or adverse outcome pathways at stress overloads, might be applied on samples daily (174).

The recently announced coordinated NIH/NCATS initiative to develop translational 'human-on-a-chip' platforms, backed by the FDA (http://www.science20.com/news_articles/biomimetics_human_body_chip-92403), has identified leading research groups that are focusing on chip-based disease model development within the USA. This is an attractive opportunity to create new research alliances among all the partners within the field, to convert this roadmap into a reality.

Realities and Fantasies Concerning 'Human-on-a-chip' Systems

Nowadays, AOPs are the result of a largely retrospective analysis of data derived from animals or humans, and informed by specific risk assessment problems. Most of the work is still descriptive or phenomenological science, followed by a certain amount of reductionism to elucidate mechanisms or develop relevant correlations which culminate in the development of synthetic generalisations or models.

Based on the developments of recent years on 'organ-on-a-chip' and short-term 'human-on-a-chip' models, hopes to find ultimate systemic replacements for the aforementioned animal tests or humans through translational 'human-on-a-chip' concepts have been raised to a historical high. Recent discoveries in human organ development and repair, progress in adult stem cell niche research, and novel insights into the interplay of vascular, stromal and immune systems, have fed that enthusiasm. We have documented that 'human-on-a-chip' systems based on technical microfluidic flow channels are already providing valuable insights into acute systemic tissue responses. Further optimisation of such microsystems and the tissues used, as discussed in this review, will make them a realistic alternative to systemic acute toxicity testing in animals.

A paradigm shift is mandatory to meet the requirements of systemic repeated dose long-term substance testing. Given an ethically acceptable supply of the necessary human tissues, by carefully weighing ethical issues against medical needs, 'human-on-a-chip' systems that provide unlimited homeostasis and organoid repair capability on the basis of biological vascularisation, physiological blood perfusion and the maintenance of organ-specific stem cell niches, could turn from fantasy to reality within this decade. A possible roadmap toward such systems has been outlined. The authors see this approach as the only biologically inspired and scientifically grounded pathway toward the full replacement of any systemic, repeated dose safety or efficacy evaluation in animals. It will remain a challenge to extend their usage to the generation of clinical phase 1 trial data. Such homeostatic 'human-on-a-chip' systems could, furthermore, offer the chance of testing properties related to a single individual tissue donor in a personalised manner. Also, the use of patient tissues may, in the future, allow for disease modelling and, consequently, efficacy testing of drug candidates in 'patient-on-a-chip' models. Such systems would be a reasonable alternative to animal disease models, but whether they would generate relevant clinical phase 2 trial data, remains pure speculation.

Perhaps, at some time in the near future, the term 'dynamic' will not only be used to describe the ability of such systems to constantly perfuse fluids and gases through homotopic or heterotopic cell aggregates over days and weeks, but will also represent:

— long-term dynamic nutrition homeostasis among groups of relevant human organoids, with steady-state protein provision by the liver lobuli, oxygen supply by lung alveoli, erythrocyte transport, and blood capillary gas exchange;

— cell and tissue homeostasis of these organoids, enabling organoid-specific cell turnover and ECM remodelling at a physiological steady-state; and

— accelerated cellular repair mechanisms leading toward the restoration of the healthy micro-organismal steady-state, a diseased steady-state, or death in response to relevant perturbations, such as pathogen invasion, tumour transformation or toxic substance exposure.

If this happens, the replacement of laboratory animals used for the safety assessment of chemicals and toxicity, and the efficacy evaluation of drug candidates, could realistically be envisaged.

Finally, it remains wishful thinking to expect such a 'homunculus' (small man) on-a-chip to

develop, for example, impaired consciousness or human-like myocardial infarction. One must always keep in mind that the term 'human' in 'human-on-a-chip' has the meaning of an artificial copy, effigy or image. The uniqueness of a human being is inviolable.

Acknowledgements

We thank www.wesapiens.org for the images used in Figure 2, Peter Mangel for the creative help with Figures 3 and 8, Mathias Busek, Sven Brincker, Alexandra Lorenz and Ulrike Menzel for help with Figure 5, and Katharina Schimek for Figure 7. Finally, the review would not exist with the outstanding creative assistance of Philip Saunders.

Received 18.09.12; received in final form 19.09.12; accepted for publication 19.09.12.

References

1. Binetti, R., Costamagna, F.M. & Marcello, I. (2008). Exponential growth of new chemicals and evolution of information relevant to risk control. *Annali dell'Istituto Superiore di Sanità* **44**, 13–15.
2. Egeghy, P.P., Judson, R., Gangwal, S., Mosher, S., Smith, D., Vail, J. & Cohen Hubal, E.A. (2012). The exposure data landscape for manufactured chemicals. *Science of the Total Environment* **414**, 159–166.
3. UN (2009). *Consolidated List of Products Whose Consumption and/or Sale Have Been Banned, Withdrawn, Severely Restricted or not Approved by Governments. Pharmaceuticals*. 14th edn, 94pp. New York, NY, USA: United Nations, Department of Economic and Social Affairs.
4. UN (2005). *Consolidated List of Products Whose Consumption and/or Sale Have Been Banned, Withdrawn, Severely Restricted or not Approved by Governments. Pharmaceuticals*. 12th edn, 598pp. New York, USA: United Nations, Department of Economic and Social Affairs.
5. Russell, W.M.S. & Burch, R.L. (1959). *The Principles of Humane Experimental Technique*, 238pp. London, UK: Methuen.
6. European Council (1986). Council *Directive 86/609/EEC* of November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal of the European Union* **L358**, 18.12.1986, 1–28.
7. European Parliament (2010). *Directive 2010/63/EU* of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union* **L276**, 20.10.2010, 33–79.
8. European Parliament (2003). *Directive 2003/15/EC* of the European Parliament and of the Council of 27 February 2003 amending *Council Directive 76/768/EEC* on the approximation of the laws of the Member States relating to cosmetic products. *Official Journal of the European Union* **L66**, 11.03.2003, 26–35.
9. European Parliament (2006). *Regulation (EC) No 1907/2006* of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending *Directive 1999/45/EC* and repealing *Council Regulation (EEC) No 793/93* and *Commission Regulation (EC) No 1488/94* as well as *Council Directive 76/769/EEC* and *Commission Directives 91/155/EEC*, *93/67/EEC*, *93/105/EC* and *2000/21/EC*. *Official Journal of the European Union* **L396**, 30.12.2006, 1–849.
10. European Parliament (1999). *Directive 1999/45/EC* of the European Parliament and of the Council of 31 May 1999 concerning the approximation of the laws, regulations and administrative provisions of the Member States relating to the classification, packaging and labelling of dangerous preparations. *Official Journal of the European Union* **L200**, 30.07.1999, 1–68.
11. Rovida, C. & Hartung, T. (2009). Re-evaluation of animal numbers and costs for *in vivo* tests to accomplish REACH legislation requirements for chemicals — a report by the transatlantic think tank for toxicology (t(4)). *ALTEX* **26**, 187–208.
12. Basketter, D., Crozier, J., Hubesch, B., Manou, I., Mehling, A. & Scheel, J. (2012). Optimised testing strategies for skin sensitization — The LLNA and beyond. *Regulatory Toxicology & Pharmacology* **64**, 9–16.
13. Hartung, T., Blaauboer, B.J., Bosgra, S., Carney, E., Coenen, J., Conolly, R.B., Corsini, E., Green, S., Faustman, E.M., Gaspari, A., Hayashi, M., Wallace Hayes, A., Hengstler, J.G., Knudsen, L.E., Knudsen, T.B., McKim, J.M., Pfaller, W. & Roggen, E.L. (2011). An expert consortium review of the EC-commissioned report 'Alternative (Non-Animal) methods for cosmetics testing: Current status and future prospects — 2010'. *ALTEX* **28**, 183–209.
14. Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Benthem, J., Zuang, V., Andersen, K.E., Angers-Loustau, A., Aptula, A., Bal-Price, A., Benfenati, E., Bernauer, U., Bessems, J., Bois, F.Y., Boobis, A., Brandon, E., Bremer, S., Broschard, T., Casati, S., Coecke, S., Corvi, R., Cronin, M., Daston, G., Dekant, W., Felter, S., Grignard, E., Gundert-Remy, U., Heinonen, T., Kimber, I., Kleinjans, J., Komulainen, H., Kreiling, R., Kreysa, J., Leite, S.B., Loizou, G., Maxwell, G., Mazzatorta, P., Munn, S., Pfuhler, S., Phrakonkham, P., Piersma, A., Poth, A., Prieto, P., Repetto, G., Rogiers, V., Schoeters, G., Schwarz, M., Serafimova, R., Tähti, H., Testai, E., van Delft, J., van Loveren, H., Vinken, M., Worth, A. & Zaldivar, J.M. (2011). Alternative (non-animal) methods for cosmetics testing: Current status and future prospects — 2010. *Archives of Toxicology* **85**, 367–485.
15. Kimber, I., Humphris, C., Westmoreland, C., Alepee, N., Negro, G.D. & Manou, I. (2011). Computational chemistry, systems biology and toxicology. Harnessing the chemistry of life: Revolutionizing toxicology. a commentary. *Journal of Applied Toxicology* **31**, 206–209.
16. Seiler, A.E.M. & Spielmann, H. (2011). The valida-

ted embryonic stem cell test to predict embryotoxicity *in vitro*. *Nature Protocols* **6**, 961–978.
17. Gibb, S. (2008). Toxicity testing in the 21st century: A vision and a strategy. *Reproductive Toxicology* **25**, 136–138.
18. Collins, F.S., Gray, G.M. & Bucher, J.R. (2009). Transforming environmental health protection. *Science, New York* **319**, 906–907.
19. Krewski, D., Westphal, M., Al-Zoughool, M., Croteau, M.C. & Andersen, M.E. (2011). New directions in toxicity testing. *Annual Review of Public Health* **32**, 161–178.
20. Dix, D.J., Houck, K.A., Martin, M.T., Richard, A.M., Setzer, R.W. & Kavlock, R.J. (2007). The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicological Sciences* **95**, 5–12.
21. Chi, K.R. (2012). Stemming the toxic tide. *The Scientist* **26**, 1–6.
22. Roep, B.O., Buckner, J., Sawcer, S., Toes, R. & Zipp, F. (2012). The problems and promises of research into human immunology and autoimmune disease. *Nature Medicine* **18**, 48–53.
23. Kirsch-Volders, M., Decordier, I., Elhajouji, A., Plas, G., Aardema, M.J. & Fenech, M. (2011). *In vitro* genotoxicity testing using the micronucleus assay in cell lines, human lymphocytes and 3D human skin models. *Mutagenesis* **26**, 177–184.
24. Zeevi-Levin, N., Itskovitz-Eldor, J. & Binah, O. (2012). Cardiomyocytes derived from human pluripotent stem cells for drug screening. *Pharmacology & Therapeutics* **134**, 180–188.
25. Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E. & Villeneuve, D.L. (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology & Chemistry/SETAC* **29**, 730–741.
26. Wetmore, B., Wambaugh, J.F., Ferguson, S.S., Sochaski, M.A., Rotroff, D.M., Freeman, K., Clewell, H.J., 3rd, Dix, D.J., Andersen, M.E., Houck, K.A., Allen, B., Judson, R.S., Singh, R., Kavlock, R.J., Richard, A.M. & Thomas, R.S. (2012). Integration of dosimetry, exposure, and high-throughput screening data in chemical toxicity assessment. *Toxicological Sciences* **125**, 157–174.
27. Rotroff, D.M., Wetmore, B.A., Dix, D.J., Ferguson, S.S., Clewell, H.J., Houck, K.A., Lecluyse, E.L., Andersen, M.E., Judson, R.S., Smith, C.M., Sochaski, M.A., Kavlock, R.J., Boellmann, F., Martin, M.T., Reif, D.M., Wambaugh, J.F. & Thomas R.S. (2010). Incorporating human dosimetry and exposure into high-throughput *in vitro* toxicity screening. *Toxicological Sciences* **117**, 348–358.
28. Hays, S.M., Aylward, L.L., LaKind, J.S., Bartels, M.J., Barton, H.A., Boogaard, P.J., Brunk, C., DiZio, S., Dourson, M., Goldstein, D.A., Lipscomb, J., Kilpatrick, M.E., Krewski, D., Krishnan, K., Nordberg, M., Okino, M., Tan, Y.M., Viau, C., Yager, J.W. & Biomonitoring Equivalents Expert Workshop (2008). Guidelines for the derivation of biomonitoring equivalents: Report from the Biomonitoring Equivalents Expert Workshop. *Regulatory Toxicology & Pharmacology* **51**, S4–S15.
29. LaKind, J.S., Aylward, L.L., Brunk, C., DiZio, S., Dourson, M., Goldstein, D.A., Kilpatrick, M.E., Krewski, D., Bartels, M.J., Barton, H.A., Boogaard, P.J., Lipscomb, J., Krishnan, K., Nordberg, M., Okino, M., Tan, Y.M., Viau, C., Yager, J.W., Hays, S.M. & Biomonitoring Equivalents Expert Workshop (2008). Guidelines for the communication of biomonitoring equivalents: Report from the Biomonitoring Equivalents Expert Workshop. *Regulatory Toxicology & Pharmacology* **51**, S16–S26.
30. Bhattacharya, S., Zhang, Q., Carmichael, P.L., Boekelheide, K. & Andersen, M.E. (2011). Toxicity testing in the 21 century: Defining new risk assessment approaches based on perturbation of intracellular toxicity pathways. *PloS One* **6**, e20887.
31. Judson, R.S., Kavlock, R.J., Setzer, R.W., Hubal, E.A., Martin, M.T., Knudsen, T.B., Houck, K.A., Thomas, R.S., Wetmore, B.A. & Dix, D.J. (2011). Estimating toxicity-related biological pathway altering doses for high-throughput chemical risk assessment. *Chemical Research in Toxicology* **24**, 451–462.
32. Clewell, H.J., Tan, Y.M., Campbell, J.L. & Andersen, M.E. (2008). Quantitative interpretation of human biomonitoring data. *Toxicology & Applied Pharmacology* **231**, 122–133.
33. Thomas, R.S., Black, M.B., Li, L., Healy, E., Chu, T.M., Bao, W., Andersen, M.E. & Wolfinger, R.D. (2012). A comprehensive statistical analysis of predicting *in vivo* hazard using high-throughput *in vitro* screening. *Toxicological Sciences* **128**, 398–417.
34. De Buck, S.S. & Mackie, C.E. (2007). Physiologically based approaches towards the prediction of pharmacokinetics: *In vitro–in vivo* extrapolation. *Expert Opinion on Drug Metabolism & Toxicology* **3**, 865–878.
35. Caldwell, G.W., Yan, Z., Tang, W., Dasgupta, M. & Hasting, B. (2009). ADME optimization and toxicity assessment in early- and late-phase drug discovery. *Current Topics in Medicinal Chemistry* **9**, 965–980.
36. McLimans, W.F., Blumenson, L.E. & Tunnah, K.V. (1968). Kinetics of gas diffusion in mammalian cell culture systems. II. Theory. *Biotechnology & Bioengineering* **10**, 741–763.
37. Knazek, R.A. (1972). Cell culture on artificial capillaries: An approach to tissue growth *in vitro*. *Science* **178**, 3–5.
38. Catapano, G. & Gerlach, J.C. (2007). Bioreactors for liver tissue engineering. *Topics in Tissue Engineering* **3**, 1–42.
39. Wang, Y., Susando, T., Lei, X., Anene-Nzelu, C., Zhou, H., Liang, L.H. & Yu, H. (2010). Current development of bioreactors for extracorporeal bioartificial liver (Review). *Biointerphases* **5**, FA116–131.
40. Giese, C., Demmler, C.D., Ammer, R., Hartmann, S., Lubitz, A., Miller, L., Müller, R., Marx, U. (2006). A human lymph node *in vitro* — challenges and progress. *Artificial Organs* **30**, 803–808.
41. Giese, C., Lubitz, A., Demmler, C.D., Reuschel, J., Bergner, K. & Marx, U. (2010). Immunological substance testing on human lymphatic microorganoids *in vitro*. *Journal of Biotechnology* **148**, 38–45.
42. Pörtner, R. & Giese, C. (2007). An overview on bioreactor design, prototyping and process control for reproducible three-dimensional tissue culture. In *Drug Testing* In Vitro*: Breakthroughs and Trends in Cell Culture Technology* (ed. U. Marx & V.

Sandig), pp. 53–78. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA.
43. Marx, U. & Sandig, V. (eds) (2007). *Drug Testing* In Vitro*: Breakthroughs and Trends in Cell Culture Technology*, 318pp. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA.
44. Marx, U. (2012). Trends in cell culture technology. In *New Technologies for Toxicity Testing* (ed. M. Balls, R.D. Combes & N. Bhogal), pp. 26–46. Austin, TX, USA & New York, NY, USA: Landes Bioscience & Springer Science+Business Media, LLC.
45. Griffith, L.G. & Swartz, M. (2006). Capturing complex 3D tissue physiology *in vitro*. *Nature Reviews. Molecular Cell Biology* **7**, 211–224.
46. Nelson, C.M. & Gleghorn, J.P. (2011). Sculpting organs: Mechanical regulation of tissue development. *Annual Review of Biomedical Engineering* **14**, 129–154.
47. Peyton, S.R., Kalcioglu, Z.I., Cohen, J.C., Runkle, A.P., Van Vliet, K.J., Lauffenburger, D.A. & Griffith, L.G. (2011). Marrow-derived stem cell motility in 3D synthetic scaffold is governed by geometry along with adhesivity and stiffness. *Biotechnology & Bioengineering* **108**, 1181–1193.
48. Dvir, T., Timko, B.P., Brigham, M.D., Naik, S.R., Karajanagi, S.S., Levy, O., Jin, H., Parker, K.K., Langer, R. & Kohane, D.S. (2012). Nanowired three dimensional cardiac patches. *Nature Nanotechnology* **6**, 720–725.
49. Ahadian, S., Ramón-Azcón, J., Ostrovidov, S., Camci-Unal, G., Hosseini, V., Kaji, H., Ino, K., Shiku, H., Khademhosseini, A. & Matsue, T. (2012). Interdigitated array of Pt electrodes for electrical stimulation and engineering of aligned muscle tissue. *Lab on a Chip* **12**, 3491–3503.
50. Bussek, A., Wettwer, E., Christ, T., Camelliti, P. & Ravens, U. (2009). Tissue slices from adult mammalian hearts as a model for pharmacological drug testing. *Cellular Physiology Biochemistry & Biochemistry* **24**, 527–536.
51. Johnstone, A.F.M., Gross, G.W., Weiss, D.G., Schroeder, O.H., Gramowski, A. & Shafer, T.J. (2010). Microelectrode arrays: A physiologically based neurotoxicity testing platform for the 21st century. *Neurotoxicology* **31**, 331–350.
52. Gramowski, A., Jügelt, K., Schröder, O.H.U., Weiss, D.G. & Mitzner, S. (2011). Acute functional neurotoxicity of lanthanum(III) in primary cortical networks. *Toxicological Sciences* **120**, 173–83.
53. Himmel, H.M., Bussek, A., Hoffmann, M., Beckmann, R., Lohmann, H., Schmidt, M. & Wettwer, E. (2012). Field and action potential recordings in heart slices: Correlation with established *in vitro* and *in vivo* models. *British Journal of Pharmacology* **166**, 276–296.
54. Park, T.H. & Shuler, M.L. (2003). Integration of cell culture and microfabrication technology. *Biotechnology Progress* **19**, 243–253.
55. Andersson, H. & van den Berg, A. (2004). Microfabrication and microfluidics for tissue engineering: State of the art and future opportunities. *Lab on a Chip* **4**, 98–103.
56. Kim, L., Toh, Y.C., Voldman, J. & Yu, H. (2007). A practical guide to microfluidic perfusion culture of adherent mammalian cells. *Lab on a Chip* **7**, 681–694.
57. Wu, M.H., Huang, S.B. & Lee, G.B. (2010). Microfluidic cell culture systems for drug research. *Lab on a Chip* **10**, 939–956.
58. Huh, D., Hamilton, G. & Ingber, D.E. (2011). From 3D cell culture to organs-on-chips. *Trends in Cell Biology* **21**, 745–754.
59. Ingber, D.E. & Whitesides, G.M. (2012). Lab on a chip: United States of America. *Lab on a Chip* **12**, 2089–2090.
60. Young, E.W.K. & Simmons, C. (2010). Macro- and microscale fluid flow systems for endothelial cell biology. *Lab on a Chip* **10**, 143–160.
61. Gu, W., Zhu, X., Futai, N., Cho, B.S. & Takayama, S. (2004). Computerized microfluidic cell culture using elastomeric channels and Braille displays. *Proceedings of the National Academy of Sciences of the USA* **101**, 15,861–15,866.
62. Leclerc, E., Sakai, Y. & Fujii, T. (2004). Microfluidic PDMS (polydimethylsiloxane) bioreactor for large-scale culture of hepatocytes. *Biotechnology Progress* **20**, 750–755.
63. Powers, M.J., Domansky, K., Kaazempur-Mofrad, M.R., Kalezi, A., Capitano, A., Upadhyaya, A., Kurzawski, P., Wack, K.E., Stolz, D.B., Kamm, R. & Griffith, L.G. (2002). A microfabricated array bioreactor for perfused 3D liver culture. *Biotechnology & Bioengineering* **78**, 257–269.
64. Lee, P.J., Hung, P.J. & Lee, L.P. (2007). An artificial liver sinusoid with a microfluidic endothelial-like barrier for primary hepatocyte culture. *Biotechnology & Bioengineering* **97**, 1340–1346.
65. Toh, Y.C., Zhang, C., Zhang, J., Khong, Y.M., Chang, S., Samper, V.D., van Noort, D., Hutmacher, D.W. & Yu, H. (2007). A novel 3D mammalian cell perfusion-culture system in microfluidic channels. *Lab on a Chip* **7**, 302–309.
66. Toh, Y.C., Lim, T.C., Tai, D., Xiao, G., van Noort, D. & Yu, H. (2009). A microfluidic 3D hepatocyte chip for drug toxicity testing. *Lab on a Chip* **9**, 2026–2035.
67. Carraro, A., Hsu, W.M., Kulig, K.M., Cheung, W.S., Miller, M.L., Weinberg, E.J., Swart, E.F., Kaazempur-Mofrad, M., Borenstein, J.T., Vacanti, J.P. & Neville, C. (2008). *In vitro* analysis of a hepatic device with intrinsic microvascular-based channels. *Biomedical Microdevices* **10**, 795–805.
68. Goral, V.N., Hsieh, Y.C., Petzold, O.N., Clark, J.S., Yuen, P.K. & Faris, R.A. (2010). Perfusion-based microfluidic device for three-dimensional dynamic primary human hepatocyte cell culture in the absence of biological or synthetic matrices or coagulants. *Lab on a Chip* **10**, 3380–3386.
69. Park, J., Li, Y. & Toner, M. (2008). Radial flow hepatocyte bioreactor using stacked microfabricated grooved substrates. *Biotechnology & Bioengineering* **99**, 455–467.
70. Ho, C.T., Lin, R.Z., Chang, W.Y., Chang, H. & Liu, C.H. (2006). Rapid heterogeneous liver-cell on-chip patterning via the enhanced field-induced dielectrophoresis trap. *Lab on a Chip* **6**, 724–734.
71. Rhee, S.W., Taylor, A.M., Tu, C.H., Cribbs, D.H., Cotman, C.W. & Jeon, N.L. (2005). Patterned cell culture inside microfluidic devices. *Lab on a Chip* **5**, 102–107.
72. Grafton, M.M.G., Wang, L., Vidi, P.A., Leary, J. & Lelièvre, S. (2011). Breast on-a-chip: Mimicry of the channeling system of the breast for development of theranostics. *Integrative Biology* **3**, 451–459.
73. Nakayama, H., Kimura, H., Komori, K., Fujii, T. & Sakai, Y. (2008). Development of a disposable

three-compartment micro-cell culture device for toxicokinetic study in humans and its preliminary evaluation. *AATEX* **14**, 619–622.
74. Hung, P.J., Lee, P.J., Sabounchi, P., Aghdam, N., Lin, R. & Lee, L.P. (2005). A novel high aspect ratio microfluidic design to provide a stable and uniform microenvironment for cell growth in a high throughput mammalian cell culture array. *Lab on a Chip* **5**, 44–48.
75. Chung, B.G., Flanagan, L.A., Rhee, S.W., Schwartz, P.H., Lee, A.P., Monuki, E.S. & Jeon, N.L. (2005). Human neural stem cell growth and differentiation in a gradient-generating microfluidic device. *Lab on a Chip* **5**, 401–406.
76. Villa-Diaz, L.G., Torisawa, Y.S., Uchida, T., Ding, J., Nogueira-de-Souza, N.C., O'Shea, K.S., Takayama, S. & Smith, G.D. (2009). Microfluidic culture of single human embryonic stem cell colonies. *Lab on a Chip* **9**, 1749–1755.
77. Smith, G.D., Takayama, S. & Swain, J.E. (2012). Rethinking *in vitro* embryo culture: New developments in culture platforms and potential to improve assisted reproductive technologies. *Biology of Reproduction* **86**, 62.
78. Huh, D., Matthews, B.D., Mammoto, A., Montoya-Zavala, M., Hsin, H.Y. & Ingber, D.E. (2010). Reconstituting organ-level lung functions on a chip. *Science* **328**, 1662–1668.
79. Kane, B.J., Zinner, M.J., Yarmush, M.L. & Toner, M. (2006). Liver-specific functional studies in a microfluidic array of primary mammalian hepatocytes. *Analytical Chemistry* **78**, 4291–4298.
80. Hwa, A.J., Fry, R.C., Sivaraman, A., So, P.T., Samson, L.D., Stolz, D.B. & Griffith, L.G. (2007). Rat liver sinusoidal endothelial cells survive without exogenous VEGF in 3D perfused co-cultures with hepatocytes. *FASEB Journal* **21**, 2564–2579.
81. Khetani, S.R. & Bhatia, S.N. (2008). Microscale culture of human liver cells for drug development. *Nature Biotechnology* **26**, 120–126.
82. Günther, A., Yasotharan, S., Vagaon, A., Lochovsky, C., Pinto, S., Yang, J., Lau, C., Voigtlaender-Bolz, J. & Bolz, S.S. (2010). A microfluidic platform for probing small artery structure and function. *Lab on a Chip* **10**, 2341–2349.
83. Ootani, A., Li, X., Sangiorgi, E., Ho, Q.T., Ueno, H., Toda, S., Sugihara, H., Fujimoto, K., Weissman, I.L., Capecchi, M.R. & Kuo, C.J. (2010). Sustained *in vitro* intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nature Medicine* **15**, 701–706.
84. Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J. & Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche. *Nature, London* **459**, 262–265.
85. Sung, J.H., Yu,J., Luo, D., Shuler, M.L. & March, J.C. (2011). Microscale 3-D hydrogel scaffold for biomimetic gastrointestinal (GI) tract model. *Lab on a Chip* **11**, 389–392.
86. Lahar, N., Lei, N.Y., Wang, J., Jabaji, Z., Tung, S.C., Joshi, V., Lewis, M., Stelzner, M., Martín, M.G. & Dunn, J.C.Y. (2011). Intestinal subepithelial myofibroblasts support *in vitro* and *in vivo* growth of human small intestinal epithelium. *PloS One* **6**, e26898.
87. Yu, J., Peng, S., Luo, D. & March, J.C. (2012). *In vitro* 3D human small intestinal villous model for drug permeability determination. *Biotechnology & Bioengineering* **109**, 2173–2178.
88. Park, J.Y., Hwang, C.M. & Lee, S.H. (2009). Ice-lithographic fabrication of concave microwells and a microfluidic network. *Biomedical Microdevices* **11**, 129–33.
89. Cui, Z.F., Xu, X., Trainor, N., Triffitt, J.T., Urban, J.P. & Tirlapur, U.K. (2007). Application of multiple parallel perfused microbioreactors and three-dimensional stem cell culture for toxicity testing. *Toxicology in Vitro* **21**, 1318–1324.
90. van Midwoud, P.M., Merema, M.T., Verpoorte, E. & Groothuis, G.M.M. (2010). A microfluidic approach for *in vitro* assessment of interorgan interactions in drug metabolism using intestinal and liver slices. *Lab on a Chip* **10**, 2778–2786.
91. Leclerc, E., Baudoin, R., Corlu, A., Griscom, L., Luc Duval, J. & Legallais, C. (2007). Selective control of liver and kidney cells migration during organotypic cocultures inside fibronectin-coated rectangular silicone microchannels. *Biomaterials* **28**, 1820–1829.
92. Guzzardi, M.A., Vozzi, F. & Ahluwalia, A.D. (2009). Study of the crosstalk between hepatocytes and endothelial cells using a novel multicompartmental bioreactor: A comparison between connected cultures and cocultures. *Tissue Engineering: Part A* **15**, 3635–3644.
93. Vozzi, F., Heinrich, J., Bader, A. & Ahluwalia, A.D. (2009). Connected culture of murine hepatocytes and human umbilical vein endothelial cells in a multicompartmental bioreactor. *Tissue Engineering: Part A* **15**, 1291–1299.
94. Pilarek, M., Neubauer, P. & Marx, U. (2011). Biological cardio-micro-pumps for microbioreactors and analytical micro-systems. *Sensors & Actuators B: Chemical* **156**, 517–526.
95. Dash, A., Inman, W., Hoffmaster, K., Sevidal, S., Kelly, J., Obach, R.S., Griffith, L.G. & Tannenbaum, S.R. (2009). Liver tissue engineering in the evaluation of drug safety. *Expert Opinion on Drug Metabolism & Toxicology* **5**, 1159–1174.
96. Schaefer, C., Spielmann, H. & Vetter, K. (eds.) (2006). *Arzneiverordnung in Schwangerschaft und Stillzeit*, 785pp. München, Germany: Elsevier GmbH and Urban & Fischer Verlag.
97. Sweeney, L.M., Shuler, M.L., Babish, J.G. & Ghanem, A. (1995). A cell culture analogue of rodent physiology: Application to naphthalene toxicology. *Toxicology in Vitro* **9**, 307–316.
98. Sin, A., Chin, K.C., Jamil, M.F., Kostov, Y., Rao, G. & Shuler, M.L. (2004). The design and fabrication of three-chamber microscale cell culture analog devices with integrated dissolved oxygen sensors. *Biotechnology Progress* **20**, 338–345.
99. Sung, J.H. & Shuler, M.L. (2010). *In vitro* microscale systems for systematic drug toxicity study. *Bioprocess & Biosystems Engineering* **33**, 5–19.
100. Shuler, M.L. (2012). Modeling life. *Annals of Biomedical Engineering* **40**, 1399–1407.
101. Viravaidya, K., Sin, A. & Shuler, M.L. (2004). Development of a microscale cell culture analog to probe naphthalene toxicity. *Biotechnology Progress* **20**, 316–323.
102. Tatosian, D. & Shuler, M.L. (2009) A novel system for evaluation of drug mixtures for potential efficacy in treating multidrug resistant cancers. *Biotechnology & Bioengineering* **103**, 187–198.
103. Sung, J.H. & Shuler, M.L. (2009). A micro cell cul-

ture analog (microCCA) with 3-D hydrogel culture of multiple cell lines to assess metabolism-dependent cytotoxicity of anti-cancer drugs. *Lab on a Chip* **9**, 1385–1394.
104. Sung, J.H., Kam, C. & Shuler, M.L. (2010). A microfluidic device for a pharmacokinetic-pharmacodynamic (PK-PD) model on a chip. *Lab on a Chip* **10**, 446–455.
105. Mahler, G.J., Esch, M.B., Glahn, R.P. & Shuler, M.L. (2009). Characterization of a gastrointestinal tract microscale cell culture analog used to predict drug toxicity. *Biotechnology & Bioengineering* **104**, 193–205.
106. Mahler, G.J., Shuler, M.L. & Glahn, R.P. (2009). Characterization of Caco-2 and HT29-MTX cocultures in an *in vitro* digestion/cell culture model used to predict iron bioavailability. *Journal of Nutritional Biochemistry* **20**, 494–502.
107. Mahler, G.J., Esch, M.B., Tako, E., Southard, T.L., Archer, S.D., Glahn, R.P. & Shuler, M.L. (2012). Oral exposure to polystyrene nanoparticles affects iron absorption. *Nature Nanotechnology* **7**, 264–271.
108. Zhang, C., Zhao, Z., Abdul Rahim, N.A., van Noort, D. & Yu, H. (2009). Towards a human-on-chip: Culturing multiple cell types on a chip with compartmentalized microenvironments. *Lab on a Chip* **9**, 3185–3192.
109. Imura, Y., Sato, K. & Yoshimura, E. (2010). Micro total bioassay system for ingested substances: Assessment of intestinal absorption, hepatic metabolism, and bioactivity. *Analytical Chemistry* **82**, 9983–9988.
110. Sonntag, F., Schilling, N., Mader, K., Gruchow, M., Klotzbach, U., Lindner, G., Horland, R., Wagner, I., Lauster, R., Howitz, S., Hoffmann, S. & Marx, U. (2010). Design and prototyping of a chip-based multi-micro-organoid culture system for substance testing, predictive to human (substance) exposure. *Journal of Biotechnology* **148**, 70–75.
111. Wu, M.H., Huang, S.B., Cui, Z., Cui, Z. & Lee, G.B. (2008). A high throughput perfusion-based microbioreactor platform integrated with pneumatic micropumps for three-dimensional cell culture. *Biomedical Microdevices* **10**, 309–319.
112. Sonntag, F., Gruchow, M., Wagner, I., Lindner, G. & Marx, U. (2011). Miniatirisierte humane organtypische Zell- und Gewebekulturen. *BIOspektrum* **17**, 418–421.
113. Baker, M. (2011). Künstliches Organ statt Tierexperiment. *Spektrum der Wissenschaft* **10**, 12–15.
114. Liao, K.H., Tan, Y.M. & Clewell, H.J. (2007). Development of a screening approach to interpret human biomonitoring data on volatile organic compounds: Reverse dosimetry on biomonitoring data for trichloroethylene. *Risk Analysis* **27**, 1223–1236.
115. Clewell, H.J., Gearhart, J.M., Gentry, P.R., Covington, T.R., VanLandingham, C.B., Crump, K.S. & Shipp, A.M. (1999). Evaluation of the uncertainty in an oral reference dose for methylmercury due to interindividual variability in pharmacokinetics. *Risk Analysis* **19**, 547–558.
116. Larrivée, B., Freitas, C., Suchting, S., Brunet, I. & Eichmann, A. (2009). Guidance of vascular development: Lessons from the nervous system. *Circulation Research* **104**, 428–441.
117. Tam, S.J. & Watts, R.J. (2010). Connecting vascular and nervous system development: Angiogenesis and the blood–brain barrier. *Annual Review of Neuroscience* **33**, 379–408.
118. Quaegebeur, A., Lange, C. & Carmeliet, P. (2011). The neurovascular link in health and disease: Molecular mechanisms and therapeutic implications. *Neuron* **71**, 406–424.
119. Pugsley, M.K. & Tabrizchi, R. (2001). The vascular system. An overview of structure and function. *Journal of Pharmacological & Toxicological Methods* **44**, 333–340.
120. Aird, W.C. (2007). Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circulation Research* **100**, 158–173.
121. Sarin, H. (2010). Physiologic upper limits of pore size of different blood capillary types and another perspective on the dual pore theory of microvascular permeability. *Journal of Angiogenesis Research* **2**, 14.
122. Rivron, N.C., Raiss, C.C., Liu, J., Nandakumar, A., Sticht, C., Gretz, N., Truckenmüller, R., Rouwkema, J. & van Blitterswijk, C.A. (2012). Sonic Hedgehog-activated engineered blood vessels enhance bone tissue formation. *Proceedings of the National Academy of Sciences of the USA* **109**, 4413–4418.
123. Luu, N.T., Rahman, M., Stone, P.C., Rainger, G.E. & Nash, G.B. (2010). Responses of endothelial cells from different vessels to inflammatory cytokines and shear stress: Evidence for the pliability of endothelial phenotype. *Journal of Vascular Research* **47**, 451–461.
124. Tammela, T. & Alitalo, K. (2010). Lymphangiogenesis: Molecular mechanisms and future promise. *Cell* **140**, 460–476.
125. Liekens, S., De Clercq, E. & Neyts, J. (2001). Angiogenesis: Regulators and clinical applications. *Biochemical Pharmacology* **61**, 253–270.
126. Adams, R.H. & Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. *Nature Reviews. Molecular Cell Biology* **8**, 464–478.
127. Iruela-Arispe, M.L. & Davis, G.E. (2009). Cellular and molecular mechanisms of vascular lumen formation. *Developmental Cell* **16**, 222–231.
128. Lazarus, A., Del-Moral, P.M., Ilovich, O., Mishani, E., Warburton, D. & Keshet, E. (2011). A perfusion-independent role of blood vessels in determining branching stereotypy of lung airways. *Development* **138**, 2359–2368.
129. Herbert, S.P. & Stainier, D.Y.R. (2011). Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nature Reviews. Molecular Cell Biology* **12**, 551–564.
130. Carmeliet, P. & Jain, R.K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature, London* **473**, 298–307.
131. Davis, G.E., Stratman, A.N., Sacharidou, A. & Koh, W. (2011). Molecular basis for endothelial lumen formation and tubulogenesis during vasculogenesis and angiogenic sprouting. *International Review of Cell & Molecular Biology* **288**, 101–165.
132. Sudo, R., Chung, S., Zervantonakis, I.K., Vickerman, V., Toshimitsu, Y., Griffith, L.G. & Kamm, R.D. (2009). Transport-mediated angiogenesis in 3D epithelial coculture. *FASEB Journal* **23**, 2155–2164.
133. Daley, W.P., Peters, S.B. & Larsen, M. (2008). Extracellular matrix dynamics in development and regenerative medicine. *Journal of Cell Science* **121**, 255–264.

134. Lu, P. & Werb, Z. (2008). Patterning mechanisms of branched organs. *Science, New York* **322**, 1506–1509.
135. Slack, J.M.W. (2008). Origin of stem cells in organogenesis. *Science, New York* **322**, 1498–1501.
136. Zaret, K.S. & Grompe, M. (2008). Generation and regeneration of cells of the liver and pancreas. *Science, New York* **322**, 1490–1494.
137. Frantz, C., Stewart, K.M. & Weaver, V.M. (2010). The extracellular matrix at a glance. *Journal of Cell Science* **123**, 4195–4200.
138. Fantin, A., Vieira, J.M., Gestri, G., Denti, L., Schwarz, Q., Prykhozhij, S., Peri, F., Wilson, S.W. & Ruhrberg, C. (2010). Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood* **116**, 829–840.
139. Lu, P., Takai, K., Weaver, V.M. & Werb, Z. (2011). Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harbor Perspectives in Biology* **3**, 1–24.
140. Badylak, S.F., Taylor, D. & Uygun, K. (2011). Whole-organ tissue engineering: Decellularization and recellularization of three-dimensional matrix scaffolds. *Annual Review of Biomedical Engineering* **13**, 27–53.
141. Rustad, K.C., Sorkin, M., Levi, B., Longaker, M.T. & Gurtner, G.C. (2010). Strategies for organ level tissue engineering. *Organogenesis* **6**, 151–157.
142. Walles, T., Biancosino, C., Zardo, P., Gottlieb, J. & Mertsching, H. (2005). Tissue remodeling in a bioartifical fibromuscular patch following transplantation in a human. *Transplantation* **80**, 284–285.
143. Mertsching, H., Schanz, J., Steger, V., Schandar, M., Schenk, M., Hansmann, J., Dally, I., Friedel, G. & Walles, T. (2009). Generation and transplantation of an autologous vascularized bioartificial human tissue. *Transplantation* **88**, 203–210.
144. Mertsching, H., Walles, T., Hofmann, M., Schanz, J. & Knapp, W.H. (2005). Engineering of a vascularized scaffold for artificial tissue and organ generation. *Biomaterials* **26**, 6610–6617.
145. Rivron, N.C., Vrij, E.J., Rouwkema, J.L.S. & Berg, A.V.D. (2012). Tissue deformation spatially modulates VEGF signaling and angiogenesis. *Proceedings of the National Academy of Sciences of the USA* **109**, 6886–6891.
146. Schanz, J., Pusch, J., Hansmann, J. & Walles, H. (2010). Vascularised human tissue models: A new approach for the refinement of biomedical research. *Journal of Biotechnology* **148**, 56–63.
147. Miller, J.S., Stevens, K.R., Yang, M.T., Baker, B.M., Nguyen, D.H., Cohen, D.M., Toro, E., Chen, A.A., Galie, P.A., Yu, X., Chaturvedi, R., Bhatia, S.N. & Chen, C.S. (2012). Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature Materials* **11**, 1–7.
148. Isenberg, B.C., Williams, C. & Tranquillo, R.T. (2006). Small-diameter artificial arteries engineered *in vitro*. *Circulation Research* **98**, 25–35.
149. Zorn, A.M. & Wells, J.M. (2009). Vertebrate endoderm development and organ formation. *Annual Review of Cell & Developmental Biology* **25**, 221–251.
150. Lindner, G., Horland, R., Wagner, I., Ataç, B. & Lauster, R. (2011). *De novo* formation and ultrastructural characterization of a fiber-producing human hair follicle equivalent *in vitro*. *Journal of Biotechnology* **152**, 108–112.
151. Preynat-Seauve, O., Suter, D.M., Tirefort, D., Turchi, L., Virolle, T., Chneiweiss, H., Foti, M., Lobrinus, J.A., Stoppini, L., Feki, A., Dubois-Dauphin, M. & Krause, K.H. (2009). Development of human nervous tissue upon differentiation of embryonic stem cells in three-dimensional culture. *Stem Cells* **27**, 509–520.
152. McCracken, K.W., Howell, J.C., Wells, J.M. & Spence, J.R. (2011). Generating human intestinal tissue from pluripotent stem cells *in vitro*. *Nature Protocols* **6**, 1920–1928.
153. Spence, J.R., Mayhew, C.N., Rankin, S.A., Kuhar, M.F., Vallance, J.E., Tolle, K., Hoskins, E.E., Kalinichenko, V.V., Wells, S.I., Zorn, A.M., Shroyer, N.F. & Wells, J.M. (2011). Directed differentiation of human pluripotent stem cells into intestinal tissue *in vitro*. *Nature, London* **470**, 105–109.
154. Scadden, D.T. (2006). The stem-cell niche as an entity of action. *Nature, London* **441**, 1075–1079.
155. Lindvall, O., Kokaia, Z. & Rando, T. (2006). Stem cells, ageing and the quest for immortality. *Nature, London* **441**, 1080–1086.
156. Jones, D.L. & Wagers, A.J. (2008). No place like home: Anatomy and function of the stem cell niche. *Nature Reviews. Molecular Cell Biology* **9**, 11–21.
157. Sigal, S.H., Brill, S., Fiorino, S. & Reid, L.M. (1992). The liver as a stem cell and lineage system. *American Journal of Physiology* **263**, G139–G148.
158. Oh, S., Hatch, H.M. & Petersen, B.E. (2002). Hepatic oval 'stem' cell in liver regeneration. *Seminars in Cells & Developmental Biology* **13**, 405–409.
159. Kloepper, J.E., Tiede, S., Brinckmann, J., Reinhardt, D.P., Meyer, W., Faessler, R. & Paus, R. (2008). Immunophenotyping of the human bulge region: The quest to define useful *in situ* markers for human epithelial hair follicle stem cells and their niche. *Experimental Dermatology* **17**, 592–609.
160. Walker, M.R. & Stappenbeck, T.S. (2008). Deciphering the 'black box' of the intestinal stem cell niche: Taking direction from other systems. *Current Opinion in Gastroenterology* **24**, 115–120.
161. Kim, C.F. (2007). Paving the road for lung stem cell biology: Bronchioalveolar stem cells and other putative distal lung stem cells. *American Journal of Physiology. Lung Cellular & Molecular Physiology* **293**, L1092–L1098.
162. Yin, T. & Li, L. (2006). The stem cell niches in bone. *Journal of Clinical Investigation* **116**, 1195–1201.
163. Martinez-Agosto, J., Mikkola, H.K., Hartenstein, V. & Banerjee, U. (2007). The hematopoietic stem cell and its niche: A comparative view. *Genes & Development* **21**, 3044–3060.
164. Can, A. (2008). Haematopoietic stem cells niches: Interrelations between structure and function. *Transfusion & Apheresis Science* **38**, 261–268.
165. Riquelme, P., Drapeau, E. & Doetsch, F. (2008). Brain micro-ecologies: Neural stem cell niches in the adult mammalian brain. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **363**, 123–137.
166. Morgan, J.E. & Partridge, T. (2003). Muscle satellite cells. *International Journal of Biochemistry & Cell Biology* **35**, 1151–1156.
167. Leri, A., Kajstura, J.A.N. & Anversa, P. (2005). Cardiac stem cells and mechanisms of myocardial

regeneration. *Physiology Reviews* **85**, 1373–1416.
168. Chaffer, C.L., Thompson, E.W. & Williams, E.D. (2007). Mesenchymal to epithelial transition in development and disease. *Cells, Tissues, Organs* **185**, 7–19.
169. Bryant, D.M. & Mostov, K.E. (2010). From cells to organs: Building polarized tissue. *Nature Reviews. Molecular Cell Biology* **9**, 887–901.
170. Thiery, J.P., Acloque, H., Huang, R.Y.J. & Nieto, M.A. (2009). Epithelial–mesenchymal transitions in development and disease. *Cell* **139**, 871–890.
171. Nieto, M.A. (2011). The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annual Review of Cell & Developmental Biology* **27**, 347–376.
172. Dellian, M., Helmlinger, G., Yuan, F. & Jain, R.K. (1996). Fluorescence ratio imaging of interstitial pH in solid tumours: Effect of glucose on spatial and temporal gradients. *British Journal of Cancer* **74**, 1206–1215.
173. Khaustova, S., Shkurnikov, M., Tonevitsky, E., Artyushenko, V. & Tonevitsky, A. (2010). Non-invasive biochemical monitoring of physiological stress by Fourier transform infrared saliva spectroscopy. *The Analyst* **135**, 3183–3192.
174. Sakharov, D.A., Maltseva, D.V., Riabenko, E.A., Shkurnikov, M.U., Northoff, H., Tonevitsky, A.G. & Grigoriev, A.I. (2012). Passing the anaerobic threshold is associated with substantial changes in the gene expression profile in white blood cells. *European Journal of Applied Physiology* **112**, 963–972.
175. Fabro, S., Schumacher, H., Smith, R.L., Stagg, R.B. & Williams, R.T. (1965). The metabolism of thalidomide: Some biological effects of thalidomide and its metabolites. *British Journal of Pharmacology & Chemotherapy* **25**, 352–362.
176. Woollam, D.H. (1965). Principles of teratogenesis: Mode of action of thalidomide. *Proceedings of the Royal Society of Medicine* **58**, 497–501.
177. Bauer, K.S., Dixon, S.C. & Figg, W.D. (1998). Inhibition of angiogenesis by thalidomide requires metabolic activation, which is species-dependent. *Biochemical Pharmacology* **55**, 1827–1834.
178. Stephens, T.D. & Fillmore, B.J. (2000). Hypothesis: Thalidomide embryopathy-proposed mechanism of action. *Teratology* **61**, 189–195.
179. Solomon, G.M. & Schettler, T. (2000). Environment and health: 6. Endocrine disruption and potential human health implications. *Canadian Medical Association Journal* **163**, 1471–1476.
180. Bassein, E. (2004). *DDT as an Endocrine Disruptor in Human and Nonhuman Test Cases*, 5pp. Boston, MA, USA: MIT OpenCourseWare.
181. Harding, A.K., Daston, G.P., Boyd, G.R., Lucier, G.W., Safe, S.H., Stewart, J., Tillitt, D.E. & Van Der Kraak, G. (2006). Endocrine disrupting chemicals research program of the US Environmental Protection Agency: Summary of a peer-review report. *Environmental Health Perspectives* **114**, 1276–1282.
182. Patisaul, H.B. & Adewale, H.B. (2009). Long-term effects of environmental endocrine disruptors on reproductive physiology and behavior. *Frontiers in Behavioral Neuroscience* **3**, 10.
183. Somia, N. & Verma, I.M. (2000). Gene therapy: Trials and tribulations. *Nature Reviews. Genetics* **1**, 91–99.
184. Edwards, S.J.L. (2004). Research ethics committees and paternalism. *Journal of Medical Ethics* **30**, 88–91.
185. Dieppe, P., Ebrahim, S., Martin, R.M. & Jüni, P. (2004). Lessons from the withdrawal of rofecoxib. *British Medical Journal* **329**, 867–868.
186. Arbor, A. (2004). Withdrawal of Vioxx casts a shadow over Cox-2 inhibitors. *Science, New York* **306**, 384–385.
187. Clair, E.W.S. (2008). The calm after the cytokine storm: Lessons from the TGN1412 trial. *Journal of Clinical Investigation* **118**, 2006–2009.
188. Stebbings, R., Findlay, L., Edwards, C., Eastwood, D., Bird, C., North, D., Mistry, Y., Dilger, P., Liefooghe, E., Cludts, I., Fox, B., Tarrant, G., Robinson, J., Meager, T., Dolman, C., Thorpe, S.J., Bristow, A., Wadhwa, M., Thorpe, R. & Poole, S. (2012). 'Cytokine storm' in the phase I trial of monoclonal antibody TGN1412: Better understanding the causes to improve preclinical testing of immunotherapeutics. *Journal of Immunology* **179**, 3325–3331.