I. Introduction

1. Cells and their natural environment

Schoefield was one of the first and foremost researchers to define the complexity of bone marrow cell microenvironment (also referred to as niche) as elaborate and dynamic systems, including multiple components, interconnected by various interaction events.[2]

In their microenvironment, the cells grow within a three dimensional (3D) architecture, comprising extracellular matrix (ECM) and constantly contact with other cell populations by factors, transported through blood and plasma (growth factors, other soluble ligands, inorganic components). The ECM plays a burgeoning role in orchestration of cell behaviour.[3] Firstly, it provides biochemical stimuli, responsible for precise regulation of cell adhesion, morphology, proliferation and metabolic activity. Acting through the cell surface receptors, the ECM is dynamically involved into the intracellular signaling molecules and pathways that regulate gene expression and define expression of particular cell phenotypes. Secondly, the ECM presents biophysical cues, such as mechanical scaffolding and ensures anchorage of the cells, which are fundamental for self-renewal and regeneration. In a higher range, the ECM imparts cellular flexibility, necessary for cells to respond towards stress, caused by motility or gravity, and it also ensures the whole structural integrity of tissue within human body. Besides these general functions of the ECM, its components are also individually tailored for various tissue types which differ both morphologically and physiologically.

Besides the ECM-cell interactions, the cells are strongly influenced by their direct interplay with other neighbouring or circulating cells. For instance, epidermal stem cells are influenced by signals from cells within the dermis, which can occur over short range, as in the case of the dermal papilla at the base of each hair follicle.[4] The other interactions defining the complex cell-cell interplay within the microenvironments are those between stem cells and osteoblasts, microvascular cell, neural cells and other immune cells.[5-8] An example that underlines the complex nature of cell-cell interaction is the dedifferentiation of a reserve cells population, which is being activated upon stem cell “ablation”. In the liver, activation of reprogramming cascades ensures hepatocytes transformation into stem-like biliary epithelial cells, which can sustain regeneration and repopulation of damaged areas.[9]

Another important parameter is the indirect communication between the cells and constituents of the niches by secretion and transport of soluble factors or with the immune cells. For instance, the interaction between human microvascular endothelial cells (HMVECs) and mesenchymal stem cells (MSCs) has been strongly pronounced when activating the wound healing mechanisms. The MSCs near the location of the wound secrete paracrine

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factors, such as vascular endothelial growth factors (VEGF), to recruit macrophages and circulating HMVECs, accelerating the wound healing process. Although this process is not very well elucidated, the recent reports suggest it involves a cascade of molecular events, ensuring migration, ECM deposition, angiogenesis, and remodelling.[10,11] In addition, the activation of the parathyroid hormone (PTH) receptor on osteoblasts by PTH increases has been reported to increase the number of MSCs and hematopoietic stem cells within the bone marrow niche.[12]

Last but not least, physical properties (shear forces, shape or stiffness) of cell surrounding can control their fate and behaviour. In the past couple of years, these interactions have been relatively well-clarified and drug compounds that orchestrate the balance between parameters, such as rigidity (e.g., bone) or elasticity (e.g., blood vessel walls) have been launched for clinical use. North et al. demonstrated that shear forces promote blood flow which could be also controlled by medicinal compounds which were shown to improve the development of zebrafish embryonic hematopoietic stem cells in experimental animal setting.[13,14] Finally, the topography and geometry of microenvironment are well-known factors that confer differences in cell shape, adhesion and even stem cell commitment towards a particular lineage.[15-20]

Restating these interactions *in vitro* requires not only different combinations of factors but also their spatiotemporal control in order to match the dynamics of stem cell–niche interactions (**Figure 1**).

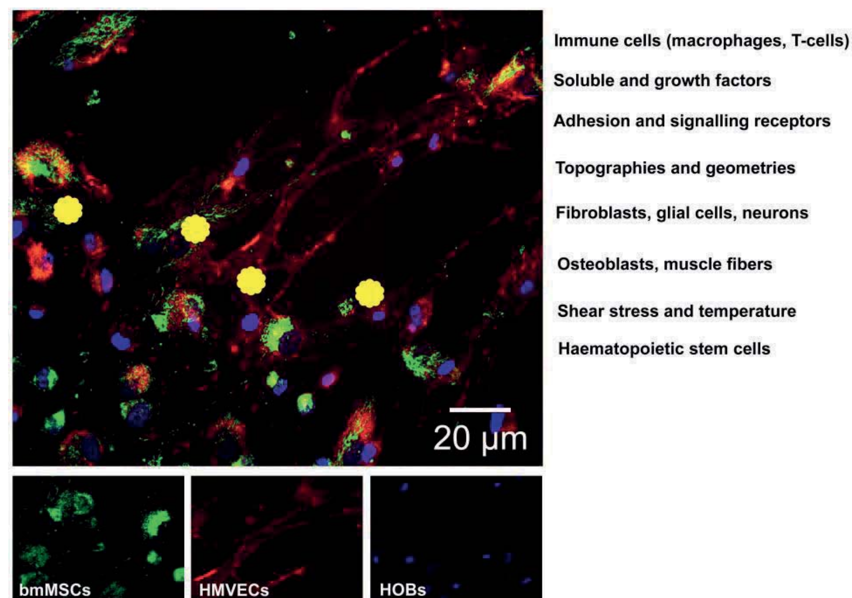

Figure 1. Basic constituents of the (stem) cell environments. The bmMSCs were stained with **MitoTracker Green®**, the HMVECs were stained with **CD31-IgG1-Alexa Fluor® 546** and the nuclei were counterstained with **HOECHST 33342.** Scale bar: 20 µm.

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2. *Microfabrication for engineering of synthetic cellular environments*

It has been almost 20 years since Langer and Vacanti reported their vision about principles of the Tissue Engineering (TE) and its basic concepts.[21,22] Since then, the biomedical applications based on TE principles span over a range of tissues after degenerative or traumatic conditions, or even diabetes and ophthalmological problems. Recent calculations predict that the market share for TE and regenerative tissue-like products would grow to about $32 billion in 2018.[23]

The microfabrication techniques have emerged as successful instrumentation for design, engineering and production of microdevices (*in vitro* platforms), which are at least partially capable of recapitulating and controlling cell-cell and cell-material interactions in 3D cell cultures, vascularization of engineered tissues for proper oxygen/nutrient delivery, or in vitro models for basic biological research.[24] There are a multitude of processes, being used to fabricate microstructures: micromachining, replica moulding (e.g. hot embossing), wet chemical processes (leaching technologies) and bonding.[25-31] These in turn have many process variations, including Rapid Prototyping, Soft Lithography, etc.[32-34]

Microfabrication processes employ various materials, such as silicon, glass/ceramics or polymers (e.g. silicone). Silicon has outstanding mechanical properties which enable design and fabrication of various micromechanical structures.[26] Glass is another material, which is particularly beneficial for biomedical applications. Firstly, glass has an excellent optical transparency, therefore is compatible with most of imaging technologies. Second of all, fused silica wafers/borosilicate glass wafers are biocompatible and have very low autofluorescence.[35] However, the latter two materials are very expensive, thus not suitable for single-use devices in biomedical research. The Polydimethylsiloxane (PDMS) elastomer is also widely used material for production of microstructures because replication can be easily performed by casting against micro- and nanometre ranges features without special equipment or clean room infrastructure[36]. Its main benefit for biomedical applications is that its surfaces can be functionalized by e.g. plasma treatment resulting in formation silanol groups, which can be further decorated with active biomolecules.[37]

For the past two decades, the polymers gained attention in microfabrication because they can be subjected to a broad spectrum of adaptable fabrication technologies and given the large number of polymeric materials available, these materials own a multitude of physical and chemical properties, including biocompatibility and biodegradability.[38] Polymers are mostly used for production of biomedical microelectromechanical system (bioMEMS) in the field of biomedical research. For the past 10 years, the bioMEMS market niche has tremendously increased, achieving a growth rate of 11.4%, which resulted in over $1 million

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of revenues for 2006 because these materials and methods are also utilized for design and creation of *in vitro* culturing scaffolds, microfluidic devices and many others.[39] The most commonly used instrumentation in the field of polymer microfabrication includes hot embossing, microscale thermoforming, injection moulding and others, described in a few excellent reviews.[25,40-42]

3. The 3D cell cultures and instructive cellular microenvironments

One of the first reports, involving culturing of cells in a 3D environment appeared in 1914 when researcher Ross Harrison cultured embryonic frog cells on various substrates and reported significant cell shapes alterations, depending on whether cells were cultured on flat substrates or 3D spider webs.[43] The 3D cell cultures have emerged as *in vitro* models which can recapitulate relevant components of complex cell–cell and cell–ECM interactions acting in a 3D communication network that maintains the specificity and homeostasis within a given tissue.[44] These interactions are essential in aspects of developmental biology, e.g. large shear forces induced by blood flow are necessary for correct heart development in zebrafish[45] or for drug screening, where reproduction of target tissue in 3D model is essential for obtaining reliable biomedical data and more organotypic response.[46] The ideal 3D cell culturing models should repeat in vitro, at least one or several relevant parameters from the natural cell environments (**Figure 2**).

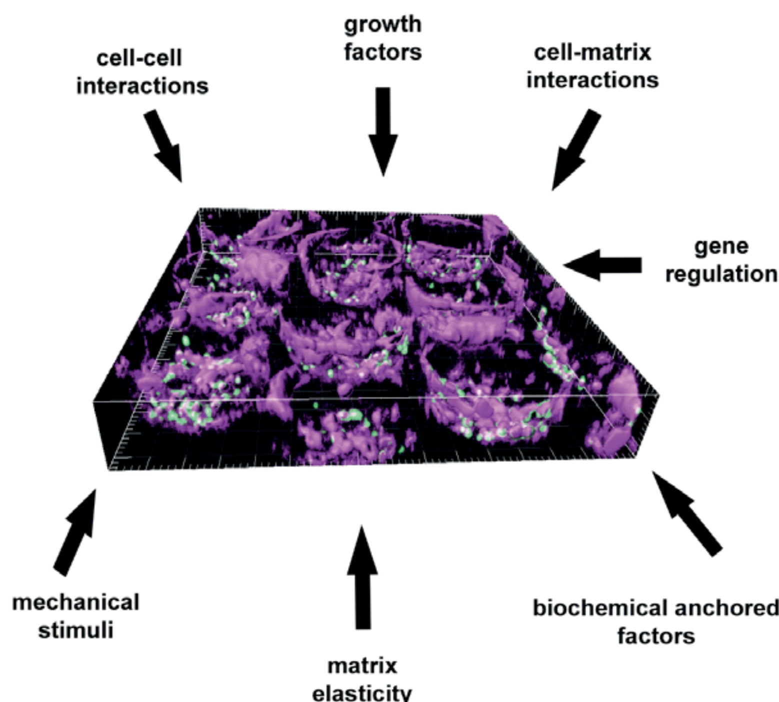

Figure 2. Towards creation of 3D culturing models.[47]

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Particularly interesting are the 3D scaffolds, known to induce changes in cell shape and cell cluster arrangement which are otherwise not observed in cells grown on 2D plastic beds, coated with individual components of the ECM.[48-50]
The strategic integration of biological principles and engineering approaches has made the 3D cultures invaluable tools to improve understanding of tumour progression and invasion and support discovery of more personalized first line treatments for cancer patients.[51-53]
Even simple processes (e.g. cell adhesion) can employ drastically different forms in 2D and 3D settings. It was demonstrated that cell migration on 2D surfaces repeatedly progresses through several steps, depending on adhesion formation and traction generation, whilst in 3D architecture, the topography, steric hindrance and anisotropic mechanics, defined by fibrous ECM regulate the migration process.[54] Notably, choosing 3D culturing platforms against conventional monolayers is advantageous when governing the maintenance of chondrocytes or governing of stem cells differentiation.[55] For instance, the 2D surfaces are non-physiological for chondrocytes because they promote an overabundance of adhesion, but exchanging the monolayer with 3D architecture is sufficient to restore chondrocyte morphology and function.[56]
The initial assumption in the field of stem cell research was that upon transplantation, the stem cells should immediately contribute to *in vivo* rebuilding of particular tissue or organ, ignoring the fact that paracrine effects in adult stem cell signalling play a major role in tissue remodelling.[57] However, the experimental results proved the complexity of these processes, also underlined by lack of biological knowledge or suitable instructive *in vitro* models. To overcome these limitations, the stem cell bioengineering employs various tools such as synthesizing novel biomaterials (e.g. hydrogels) for stem cell culture, fabricating 3D scaffolds with microscale or nanoscale topographies, micropatterning ECM in two dimensions, and performing high-throughput ECM microarrays.[58] For example, hydrogels can be tuned to reproduce e.g. the stiffness of healthy and pathological tissue and thus control differentiation and self-renewal of stem cells.[59] High-throughput ECM microarrays allow for efficient screening of effects which individual ECM components pose to stem cell fate.[60,61] Another useful approach to understand the effect of ECM on stem cells is the use of micropatterned islands, harbouring various ECM components or nano- and micro-topographies by using microcontact printing (μCP) of self-assembled monolayers.[15,62,63] Noteworthy, the patterning approaches can be applied to study the effects of cell-cell interactions or even to generate spatiotemporal resolution for growth factors, tethered to the ECM.[58,64] The generation of 3D instructive microenvironments to probe stem cell fate has also emerged as a challenging task. The synthetic 3D scaffolds require mimicking of the mechanical and biological properties of the ECM, such as ligand presentation, nano-topography, substrate elasticity, growth-factor binding, degradation and remodelling. Some of the limitations include non-

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uniform cell distribution upon inoculation or difficulties to achieve biofunctionalization of geometrically defined surfaces.[65,66]

Besides scaffolds, the growth factors are also a major constituent of an instructive microenvironment. Typically, growth factors do not act in an endocrine fashion but rather exhibit short-range diffusion through the ECM and act locally owing to their short half-lives and slow diffusion. The ultimate response of a target cell to a given factor is governed by the ability of the factors to bind to ECM, ECM degradation and growth factor concentration and cell target location.[67] In addition to localized delivery of a growth factor, simultaneous or sequential delivery of multiple growth factors has also been exploited to enhance the therapeutic efficiency by using complex polymer systems showing distinct release kinetics for growth factors.[68] There are currently several strategies for presentation of growth factors on engineered microstructures. On one hand, one could perform chemical mobilization (covalent, non-covalent) of the growth factor on or into the ECM.[69-71] On the other hand, physical encapsulation of growth factors in the delivery system is carried out.[72] The efficacy of both strategies can be significantly enhanced by 3D patterning of the growth factors on scaffolds. Furthermore, matrix degradation and subsequent diffusion-based delivery systems with pre-defined kinetics are suitable for growth factor/morphogen delivery, as they can provide sustained release in a time-dependent manner, for example for bone tissue engineering.[73] The potential applications of such tailored devices (harbouring biomimetic scaffolds with controlled release of growth factors) are drug-delivery systems in which cleavage can be initiated by small drugs, antigens or antibiotics.[74] For instance, the enhancing of encapsulated growth factors stability would allow release for extended times (e.g. months). In addition, the determination of appropriate structure of delivery materials would permit multiple factor release, with distinct kinetics.

4. *Trends, perspectives and challenges*

The latest developments in the stem cell bioengineering compensate some drawbacks by e.g. creating models with covalent coupling and selective removal of functional moieties from the biomimetic scaffolds, leading to well-orchestrated spatiotemporal control over stem cell behaviour.[75] In 2013, Sasai opened the perspectives towards the four dimensional (4D) stem cell biology where the researcher should be able to manipulate the stem cell fate through tailoring the multiple cell interactions resulting in formation of self-organized complex structures (**Figure 3**).[76,77] The latest studies in this direction include self-organization of embryonic stem cells, resulting in formation of optical cups (and other neuroectodermal structures) or development of endodermal tissue-like constructs, such as liver and pancreas.[78,79] A perspective of the engineered microenvironments would be their adaptation to clinical use, requiring multi-disciplinary approaches that combine medicine, chemistry,

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engineering and pathology to develop effective strategies for treatment of medical conditions.[80]

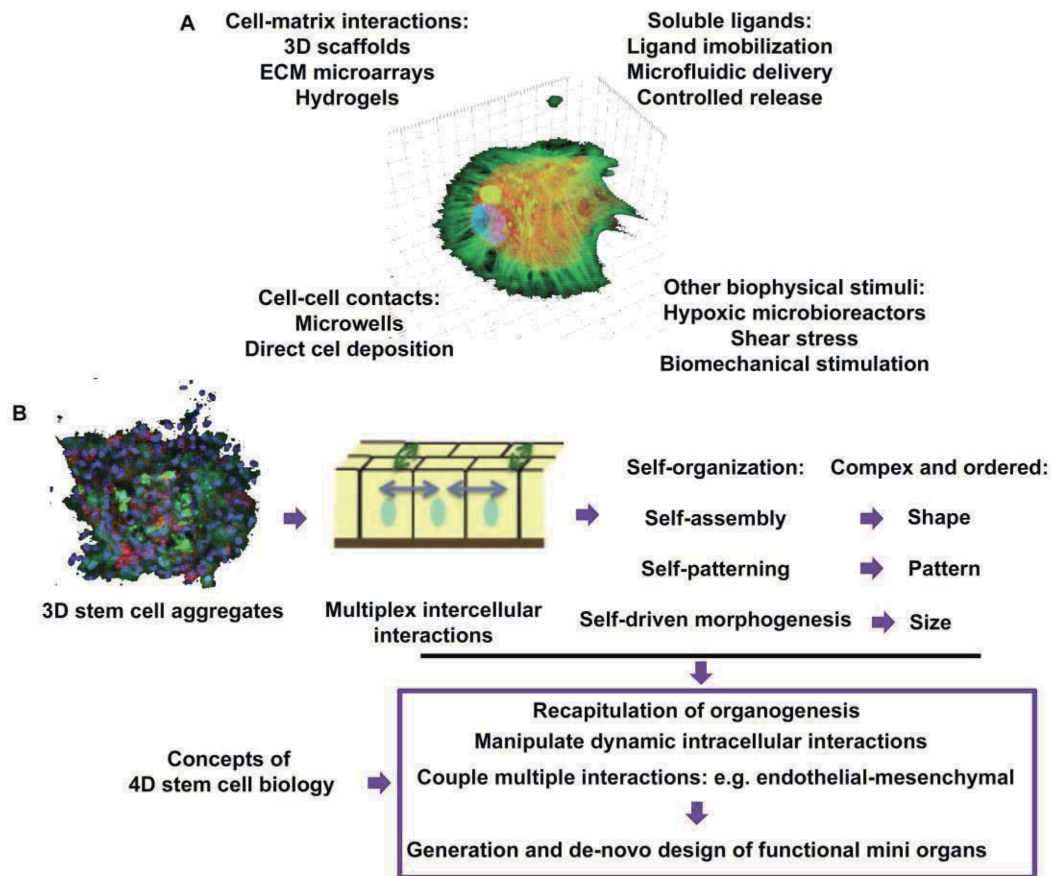

Figure 3. Engineering instructive 3D microenvironments for stem cells and concepts of 4D biology. (A) The scheme provides overview of various stem cell engineering approaches towards restating instructive scaffolds for directing of stem cell fate. (B) In 3D self-organizing cultures, progenitors induced by differentiation conditions (e.g., neural or osteogenic induction) undergo multiple cellular interactions. These local interactions result in collective behaviour leading to self-organization of complex structures. The 4D stem cell biology should aim at controlling these complex cellular interactions, and thereby manipulating the characteristics in emergent phenomena such as pattern, shape, and size. A long-term goal may be *de novo* design and formation of miniaturized organs or organ-like constructs.[65,77]

Further on, a major problem in TE is caused by the lack of simple processes to form complex tissue-constructs and different strategies have been utilized to improve the existing or engineer more efficient models: (1) direct injection of (stem) cells into the tissue of interest or the systematic circulation, (2) implantation of cells upon their organization into 3D tissue-like construct, and (3) scaffold-based delivery of signalling molecules such as medicinal compounds or growth factors, which stimulate cell proliferation and regeneration.[81] The 3D

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microfabricated scaffolds can be applied not only for *in vitro* culturing of cells in order to decipher fundamental biomedical mechanisms, but they also open up the opportunity to create constructs of clinically relevant sizes and shapes. One of the unsolved problems for design and creation of 3D scaffolds is the limited accessibility of the material for surface modification or functionalization in three dimensions. Moreover, the applicability of such systems for regenerative purposes faces several hurdles, including inhomogeneous cell distribution, formation of necrotic cores due to limited nutrient supply and last but not least lack of vascularization which hampers remodelling capacity.[82] To overcome these shortcomings new methods have been developed. For instance, bottom-up approaches have the potential to construct large complex tissue-similar constructs with defined properties including spatial and temporal control at cellular level.[83] One could use either scaffold-free culture where cells are aggregated into 3D tissue aggregates[84] or utilize gel-like materials and thus overcome the problem of compaction by allowing the cells to reside in ECM-like environment.[85] Furthermore, the 3D scaffolds can be integrated into microfluidic devices which are continuously perfused and therefore provide control over many system parameters. For example several models ensure control over fluid flow and cell patterning, which are fundamental for cardiac tissue formation or for recapitulating the villus shape of intestine.[86, 87] Several significantly funded projects have recently been concentrated in this direction, including the European Union (EU) project “The Body-on-a-Chip” focusing on the effects of interactions between drugs and their metabolites in various organ microtissues, aiming at designing platforms for personalized and patient-specific therapeutics.[88,89] In 2008, Macchiarini et al. demonstrated successful transplantation with a reengineered trachea as donor trachea was first decellularized, followed by scaffold inoculation with MSCs, triggered into chondrogenic commitment in a rotating bioreactor.[90] In 2014, Bredenkamp et al. reported a protocol for genetic transformation of fibroblasts into thymic epithelial cells, which upon transplantation established complete, fully organized thymus. Their findings provide evidence that cellular reprogramming approaches, together with suitable *in vitro* culturing platforms, can be used to generate an entire organ, thus facilitating thymus transplantation in immunosuppressed patients.[91]

Although enormous advances have been accomplished, at the time being there is not an ideal *in vitro* platform which can recapitulate at least the most relevant properties of natural (stem) cell niches and be applied for the needs of TE or tissue regeneration therapeutics.

Firstly, in contrast to differentiation *in vitro*, during embryonic development, stem cells reside in a complex 3D niche continually interacting with ECM and other cellular or biochemical components through mechanisms that are not very well elucidated. Moreover, up to now the protocols for efficient and directed differentiation of pluripotent stem cells into mature phenotypes in *in vitro* settings do not provide efficient reproducibility due to donor variations

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or biological heterogeneity.[92-96] Importantly, some recent reports revealed that bmMSCs or neural stem cells, expanded *in vitro* can acquire chromosome abnormalities, resulting in malignant transformation, underlined by still unidentified mechanisms.[97,98] Furthermore, transplantation of e.g. umbilical cord blood products as stem cell source is not only hampered by the limited number of stem cells in each infusion, but also by insufficient knowledge of the mechanisms of immune reconstitution or T-cells originated from engrafted stem cells.[99]

It is worth noting that replication of relevant physiologic conditions into an engineered scaffold requires initial correction of the relative size/shape of scaffold and tissue-like constructs. For instance, the organ size does not always scale proportionally with the body mass, but it rather obeys a number of different physical power laws, tackling e.g. the linear dimensions of an organ or tissue.[100] It is still a major challenge to choose the size (to scale-up) which provides the appropriate relative organ functional activity. As the scaffold dimensions need to be scaled accordingly, so must be the medium exchange and medium flow through the scaffolds and cellular constructs. To fulfil that requirement, one would need highly instrumented microbioreactors running in a manner that enables paracrine and endocrine signaling. Such complex platforms require low-volume pumps and miniature valves. It is a tremendous tackle to set-up such systems at low cost, with compact and affordable support hardware to allow massively parallel experiments over extended time, necessary for e.g. stem cell differentiation.[101] Recent reports demonstrated that microbioreactors, providing medium perfusion through the forming tissue-constructs, can permit moderate scale-up (μm to cm ranges) of bone cultures to clinical sizes. However, perfusion conditions require a mechanical stability of scaffolds and possibility to integrate uniform scaffold porosity with suitable geometry to facilitate flow-stimulation, which is a challenging technical task.[102,103]

Besides the scale-up considerations, the *in vitro* cultured cells are sensitive to the mechanical properties of the substrate they are maintained on, and possible variations in their stiffness or oxygen permeability. As mentioned before, the PDMS has rapidly turned into a material of choice when creating bioinspired culturing models, however it causes many issues, e.g. due to the fact that it is gas-permeable, and thus may impede the cradle of hypoxic conditions within the cell aggregates.[104] Synthetic polymers are also widely used (e.g. polylactic acid (PLA)), but they suffer from significant drawbacks, such as non-linear degradation profiles or non-natural mechanical properties.[105] Several improvements in this direction have already been achieved, e.g. incorporation of neuro-active small molecules into polymer films, which can induce enhanced neurite outgrowth.[106] However, a major hurdle is the ability to produce large quantities of such constructs as would be required for their usage as a bulk material for microfabrication of instructive scaffolds.

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Importantly, the planar surfaces of polymers, which are usually used for fabrication of microdevices, do not restate the 3D display of diverse ligands, to which cells respond in their native environments. Moreover, *in vitro* cultured cells are known to react in a sensitive manner towards spatial distribution of signaling ligands or growth factors, within 3D scaffolds.[20,107] Creating patterns of biomolecules such as proteins on planar polymer substrates, which are then subjected to mechanical structuring, pose a risk of ligand inactivation and degradation thus remains a major tackle.[108] Particularly, many substrate surfaces have relatively low accessibility, interfering with the methods for patterned surface functionalization and modification.[82,109]

Furthermore, single cell analysis has recently gained interest, mainly because it provides a solution for deciphering and understanding the mechanisms, underscoring the stem cell fate, probed in instructive environment or effects that a certain drug compound poses on individual cell behaviour. Fabrication and application of such platforms also face a number of problems, including analytical challenges (small absolute amounts of RNA/DNA for analysis, etc.), purification and separation challenges (e.g. in case of heterogeneous cell populations) and handling issues (e.g. the most microfluidic single cell platforms are designed for cell analysis, but not for prolonged culturing of cells in well-defined microenvironments).[110-112]

Withal, once a 3D platform is established, the researcher is confronted with difficulties to quantify relevant cellular processes, such as transports, secretion, and metabolic activity, or to perform real time live cell analysis over a prolonged period of time. At the same time a difficulty of sampling luminal contents is posed and harvesting of cellular components for downstream analysis (e.g. proteomic and transcriptomic assays) or complexity of such platforms impairs non-disruptive live cell analysis (e.g. fluorescent microscopy).[89]

Last but not least, creation of complex tissue-like constructs, harbouring two or more cell populations is often challenged by nutrient deficiency. A possible solution could be the incorporation of asymmetry or high aspect ratio features which sterically hinder cell compaction or one could enable the vascularization of the constructs at very early stage. However, it still remains a challenge to control the shape and size of vascularized constructs, as well as to control the differences in the composition of the liquids perfused through capillaries (or even veins), blood vessel pressure and surrounding atmosphere conditions.[113]

Although the enormous potential of engineered 3D artificial microenvironments is widely recognized, it will take quite some time to create and validate appropriate platforms, allowing for applicable and reliable assays. A closer collaboration between fields of chemistry, engineering and biomedical sciences is expected to widen the opportunities for design, fabrication and functionalization of biomimetic models or applying and optimizing bioreactor systems, which operate in user-friendly, robust manner.[114]

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In conclusion, choosing the "the most appropriate" material, together with the associated suitable functionalization procedure and microfabrication technique, would therefore require (1) expanding the set of compatible materials and their (bio-) chemical functionalization and (2) development of new fabrication methods to enable reproducible fabrication of complex microstructures. To fulfil these requirements, Giselbrecht and Truckenmüller developed **S**urface **M**odification **A**nd **R**eplication via **T**hermoforming technology (SMART) as a toolbox which combines polymer functionalization and microfabrication in sequential steps.

5. Microthermoforming and SMART technology

There have been numerous methods for polymer micromoulding for the past few decades.[115] Some of the frequently used techniques includes Microinjection moulding[116], Hot embossing[117] or casting of PDMS.[118] Thermoforming is a macroscopic process of shaping a heated thermoplastic polymer film (or a plate) by 3D stretching. A complete overview of all thermoforming variations and processes has been reviewed by Throne.[40]
The microscale thermoforming concept was developed at Karlsruhe Institute of Technology by Roman Truckenmüller and also applied in our laboratory.[119] Initially, the film is clamped around the forming zones, resulting in thinning of the polymer film compared to its initial thickness. Secondly, the film is heated and consequently softened to entropy or rubber-elastic states. Consequently, the heated film is formed by compressed nitrogen, until the film surface replicates the thermoforming mould. In the last steps, the film is cooled down (by conduction via the mould surface) below its softening range in order to retain its 3D shape. Finally, the 3D structure is demoulded (**Figure 4**).

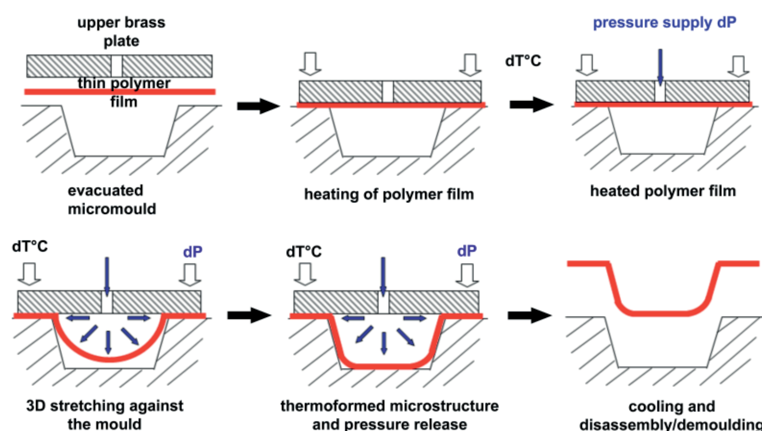

Figure 4. Main steps in microscale thermoforming. The thin polymer film is positioned in a brass tool and heated to a rubber-elastic state. The heated film is then formed by compressed nitrogen against a brass (or polymeric mould) pattern. After cooling down, the 3D shaped microstructured are demoulded.

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The microthermoformed microdevices have essential benefits, as compared to otherwise moulded microstructures, systematized in the table below (the descriptions are adapted from Truckenmüller et al[120]):

Properties	Potential applications
Thin walls	Hermetically closed microfluidic point-of-care devices that can be accessed by simple peeling or punching
Small material quantities	Expensive biopolymers or biodegradable human implants
High flexibility	Implantation under the skin or in soft tissues, disposable microfluidic chips
Low heat resistance	Micro-heat exchangers, chip-based PCR
High permeability for gases, liquids or solid particles	3D culturing platforms with porous scaffolds
Low light absorption and background fluorescence	Facilitates imaging of in vitro cell cultures within microchips or microchannels

The SMART process, developed by Giselbrecht and Truckenmüller, is based on microthermoforming and can be applied for the fabrication of thermoformed film microdevices with micro- or nano-patterned modifications of the film surface or bulk.[119,121] The pre-modifications of polymer films are usually carried out prior to thermoforming on their highly accessible planar surfaces which eases up functionalization of biochemical, physical or even biological nature. For instance, defining of the modification sites is usually performed by highly anisotropic, directed lithographic processes (**Figure 5**).

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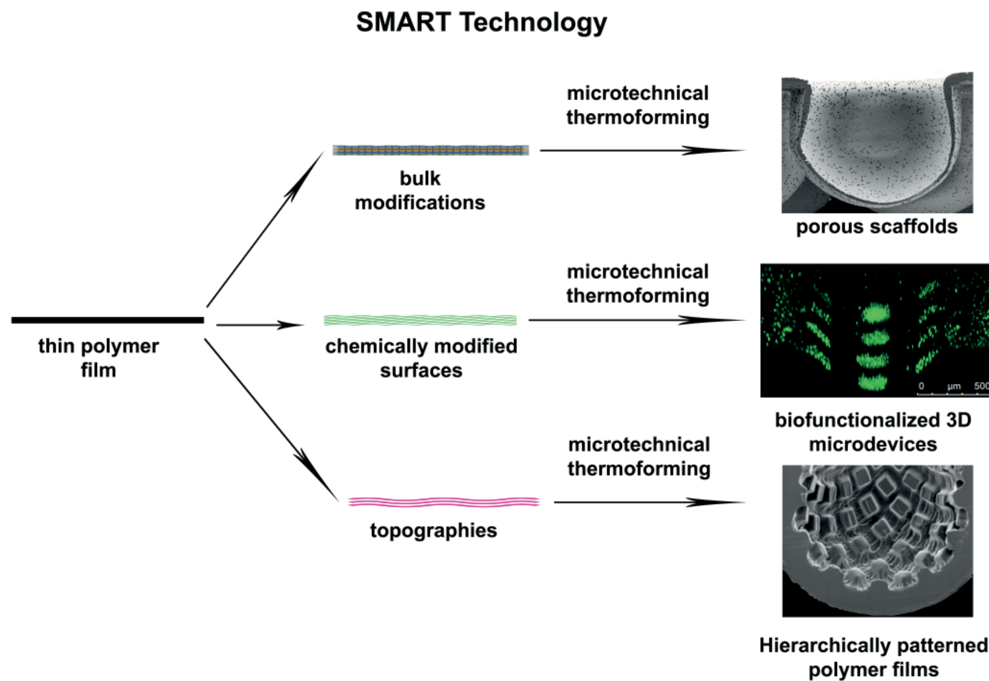

Figure 5. Development of different SMART modules. After achieving bulk modifications (heavy ion treated PC films), the films are microthermoformed and subjected to wet chemical etching to obtain an equally porous scaffolds (upper right image).[122] After performing chemical modifications on non-planar COP films (by LiPAP or CVD methods), the COP films were thermoformed and then subjected to post-process decoration with Fibronectin to achieve a 3D biofunctionalized surfaces (middle right image).[69] Initially, a thin non-planar polymer film is subjected to UV-lithography and then subjected to thermoforming, where the latent lithographic image is preserved. Finally, the wet chemical development is applied to achieve overlaid lithographic patterns.[123]

One of the first and foremost references demonstrating the potential application of SMART technology is fabrication and modification of KITChips, where high-pressure forming in the form of negative forming was used.[124,125] One trend in the development of SMART processes includes new processes for generation of micro- and nanotopographies on the surfaces of thermoformed (PMMA) films. Here, a combination of microthermoforming with nanoimprinting is used, as reported by Reinhardt and Giselbrecht et al.[126]

Another potential trend could be the hybrid integration of microthermoformed devices with devices replicated by microinjection moulding or hot embossing. Such combination would merge benefits of the flexible and adaptable thermoformed microdevices with the rigidity and shape definition of injection-moulded or embossed structures. For instance, thermoformed porous cell culture chip could be integrated in the bottoms of injection-moulded inserts for multiwell plates, comparable to Transwell® membrane inserts. A similar device was reported by Hebeiss et al., where a porous microchannel was bonded to a Boyden chamber insert and used to investigate the transendothelial transport of peptoids.[127]

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II. Aims of the thesis

This thesis aims at developing novel SMART modules for fabrication and (bio-)-functionalization of 3D scaffolds, such as porous chips (sizes ranging from a single cell resolution to 500 μm) and microchannels, followed by establishment of protocols for their biological characterization and validation. In the long-run, the goals of microfabrication processes, based on SMART technology, are development of 3D *in vitro* culturing platforms, comprising relevant microenvironment units (topographies, tethered bioactive cues, etc.) that recapitulate essential tissue-level functions and can orchestrate the fate and behaviour of (stem) cells.

In the first part of the thesis, a novel straightforward SMART module for fabrication of polymer microarrays with four basic geometries was developed. The rich information obtained from single-cell analysis can result in better understanding of the molecular machinery of a cell and its role in a complex environment. It was shown that environmental physical properties, such as geometry and rigidity act as mechanical signals which regulate cell morphology. We hypothesize that adjusting of cavity shapes and dimensions (depth, radii of curvature) would result in single cell dispersion across microarrays and may influence the cell morphology. We will investigate whether incorporation of porous scaffolds would contribute to formation of small-size aggregates, constrained by the cavity shapes, which can be maintained over prolonged time and analysed by qualitative live-cell imaging methods.

Single cell cultures are mainly useful to determine how cells attach, grow, and change their morphology as a respond to external or genetic stimuli. However, native organs and tissues consist of millions of cells, residing within specific architecture. It has been reported that formation of (stem) cell aggregates triggers morphogenic events, thus provides unique opportunities to study biomechanics in parallel with changes in pluripotent capacity and cell fate.[128,129] In the next units of this work, we developed a novel SMART module for fabrication of polymer microarrays, harbouring round geometries and porous scaffolds. We postulate that bmMSCs cultured in KIT 0.5 chips retain their stem cell phenotype; therefore these synthetic platforms can be complementary to the conventional systems for culturing bmMSCs *in vitro*. Moreover, we hypothesized that combination of biochemical (morphogens and growth factors) and biophysical cues (flow stimulation through microbioreactor development) would recapitulate and influence the regulation over relevant stages of regenerative potential of bmMSCs.

Besides their multicellular structure, native tissues consist of different cell populations, e.g. interactions between bmMSCs, osteoblasts and microvascular endothelial cells underline cellular interactions to model human osseous tissues and their vasculature.[130] Notably, three-dimensional co-cultures of osteoblasts and breast cancer cells were shown as more “organotypic” model, restating osteolytic and osteoblastic stages from metastatic

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cascades.[131] To address some of these interactions, we investigated whether the co-cultures of bmMSCs/HMVECs and bmMSCs and tumour-derived exosomes, maintained in 3D scaffolds with instructive microenvironments, might restate basic interactions within the perivascular stem cell niche *in vitro*.

Last but not least, we developed a SMART module for fabrication of straightforward assembly of multicomponent microbioreactors, comprising scaffolds with channel geometries. In addition, we hypothesized that microthermoforming, combined with surface patterning methods could generate scaffolds with well-defined geometries, incorporating robust biofunctional surfaces, created by chemical patterning and decoration. We hypothesize that these engineered microenvironments would influence cell behaviour (adhesion, viability) (**Figure 6**).

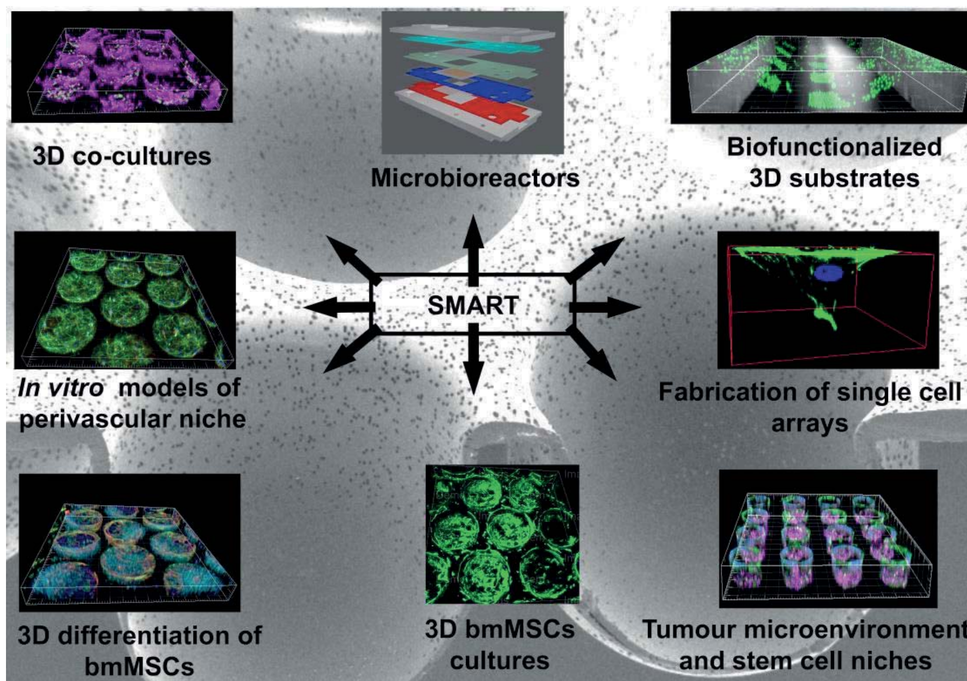

Figure 6. Aims of the thesis: development of SMART modules for fabrication of synthetic scaffolds for three-dimensional culturing and co-culturing of cells.

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