Engineered three-dimensional microenvironments as functional in vitro models of stromal tissues

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Elia Piccinini

aus **Italien**

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auf Antrag von

Prof. Ivan Martin

Prof. Antoine H.F.M. Peters

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Prof. Dr. Martin Spiess
The Dean of Faculty

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The scientist, by the very nature of his commitment, creates more and more questions, never fewer. Indeed the measure of our intellectual maturity, one philosopher suggests, is our capacity to feel less and less satisfied with our answers to better problems.

G.W. Allport

The difficulty in most scientific work lies in framing the questions rather than in finding the answers

(***A.E. Boycott***)

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Introduction

Cell culture models and current limitations

Culturing cells in a controlled environment (*in vitro*) is an invaluable resource for bio-scientists. Directly observing, stimulating, and analyzing viable cells *in vitro* often represents the only way to unveil biological mechanisms underlying cellular functions. Starting from the first pioneering approaches of the late XIX century aimed to culture cells extracted from vertebrate organisms, much advancement occurred, allowing today the culture of almost any cell type *ex vivo*. It was believed for a long time that cells required substantially a defined mix of soluble factors to be properly cultured in two-dimensional (2D) substrates mainly constituted by glass or plastic. However the use of these traditional techniques, eventually under simplistic assumptions or due to lack of alternatives, have now to face an increasing number of evidences that argue against an over-simplistic approach [1,2]. In fact, in most cases 2D substrates lack any biological resemblance when compared to the site of origin of the cell in vivo (Fig. 1a,b) [2,3].

Over-simplified in vitro models cannot provide the cells with the complex regulatory mechanisms arising, e.g., from the contact with the extracellular matrix, the cross-talk with other regulatory cells, the functional spatial organization of the cells in each tissue, and the physical stimuli derived from the stiffness and the mechanical solicitations of the surrounding microenvironment [4,5]. Co-culture systems of different cell types, e.g. the use of feeder layer for the culture of hematopoietic cells in vitro, solved only partially the limitations listed above; however, it was demonstrated that an increase in

the complexity of the culture system could be beneficial in reproducing the tissue microenvironment responsible of the maintenance of cells in vivo [6–9].

It was only in the last decades that advances in many scientific areas, e.g. cell biology, biomedicine, developmental biology, tumor biology, toxicology, biomaterial science, and bioinformatics, produced an increasing amount of data evidencing how the choice of a cell culture system can influence cell phenotype and function. The multidisciplinary aspect of Tissue Engineering (TE) has accelerated the process of bringing together scientists with different backgrounds with the common interest of developing culture systems that could allow the isolation, growth, manipulation, and use of relevant cell populations.

A central approach that was proposed is based on the mimicking in vitro of the three-dimensional (3D) spatial organization of the cells within its native tissue (Fig. 1 c,d). It was soon realized that implementing this technique posed many scientific challenges, or opportunities, to study cells in unprecedented ways. An easy technique that allows to culture in 3D relies on the intrinsic capacity of some cells to aggregate or to expand clonally in aggregates, as it is the case for spheroids or pellet culture [10]. This type of methodologies can be implemented in a relatively simple manner, also considering the number of marketed specific devices like, e.g., ultra-low adherent petri dishes and hanging drop multi-well plates. However, these culture systems do not easily allow to fine-tune the microenvironment to which the cells are exposed, thus lacking the desired control needed for some aspects of basic research, and have limited perspective scalability, therefore dramatically reducing their interest for clinical applications [11].

An alternative approach to 3D culture implies the use of biomaterials that can be used in combination with the cells [4]. In general terms, biomaterials can be derived from

natural biological sources (proteins, polysaccharides), synthetic compounds assembled in polymers, metals, ceramics, glasses ecc… Critical features of biomaterials for clinical applications are: (i) lack of cytotoxic effects, (ii) absence or limited inflammatory effect, (iii) established biodegradation rate or complete non-biodegradability.

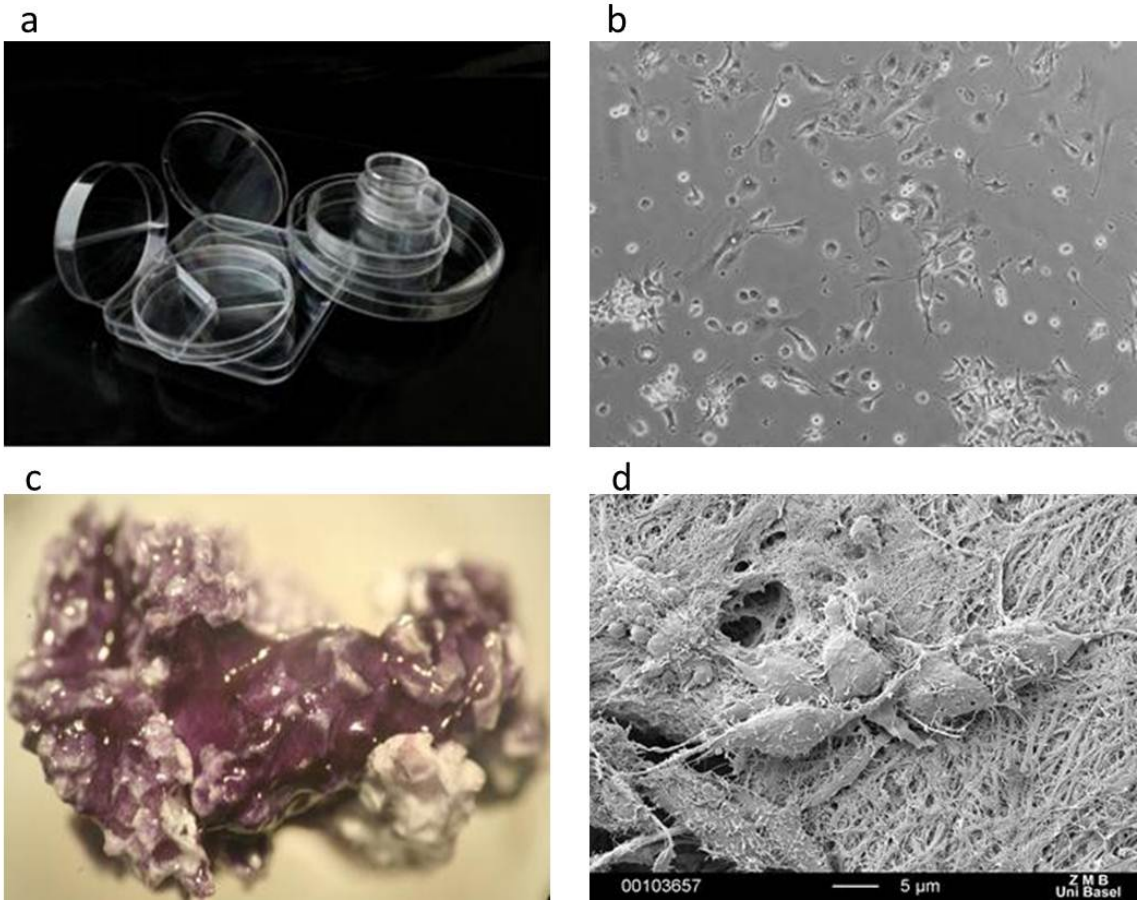

Fig.1

Petri dishes used for traditional 2D cell culture (a). Cells cultures on 2D surfaces modify their shapes to adapt to the flat, stiff, matrix-free environment (b). Viable-cells staining on a 3D scaffold made of ceramic granules coated with a fibrin matrix, cells are in purple (c). SEM image of cells growing on the surface of the granules, embedded in the proteinaceous matrix and establishing a 3D architecture with each other (d).

The use of specific material in biomedical applications is nowadays a consolidated and expanding clinical practice, e.g. through the use of bone prosthesis, heart valves, dental implants, plastic surgery, artificial ligaments, and others. However, biomaterial science has more recently become tightly associated also with fundamental research, providing new tools to investigate cell biology. The last years have witnessed a tremendous increase in the number of techniques aimed at precisely tune the features of the biomaterials both in physical and chemical terms [12].

As a result, bulk composition of the material is not anymore the only parameter to consider when a substrate for cell culture has to be chosen: macroscopic architecture, nanostructure, porosity, pore connectivity, stiffness, elasticity, accessibility of membrane-bound molecules to competent protein motives to control adhesion, ligand presentation, protein adsorption, controlled release of factors, and biodegradation rate are some of the aspects that can be tuned in the final product.

Material properties can influence cell behavior through multiple mechanisms. In particular, the possibility of culturing cells in a 3D matrix opens up the possibility to mimic the physiological spatial relationships that govern cells in vivo. In fact, most of the organs, glands and structures in vivo have a defined 3D shape and confinement that are defined by the stroma, a supportive framework of mesodermal origin usually composed of cellular connective tissue responsible of providing the necessary microenvironment to instruct and maintain tissue specific cells.

As an example, while bones have the structural function of supporting the body and protecting soft organs, they also act as a specialized microenvironment that enables hematopoietic stem cells (HSC) to maintain a life-long production of differentiated blood cells. Similarly, the stroma of the thymus is mainly represented by a complex 3D

network of epithelial cells that constantly survey developing thymocytes applying a tight control on the compliance of their unique T cell receptor (TCR).

Despite the theoretical enunciation of Schofield in 1973 about the concept of the stem cell niche (Fig. 2) contained already the indication that cells require specific interactions with neighboring cells providing the necessary functional microenvironment, attempts to culture functional cells in vitro on 2D rigid substrates has often neglected the complexity of the chemo-physical complexity of the original in vivo site. Moreover, the same concept of inadequacy described above for carrying out stem cells culture is valid also for cells of the stromal compartment.

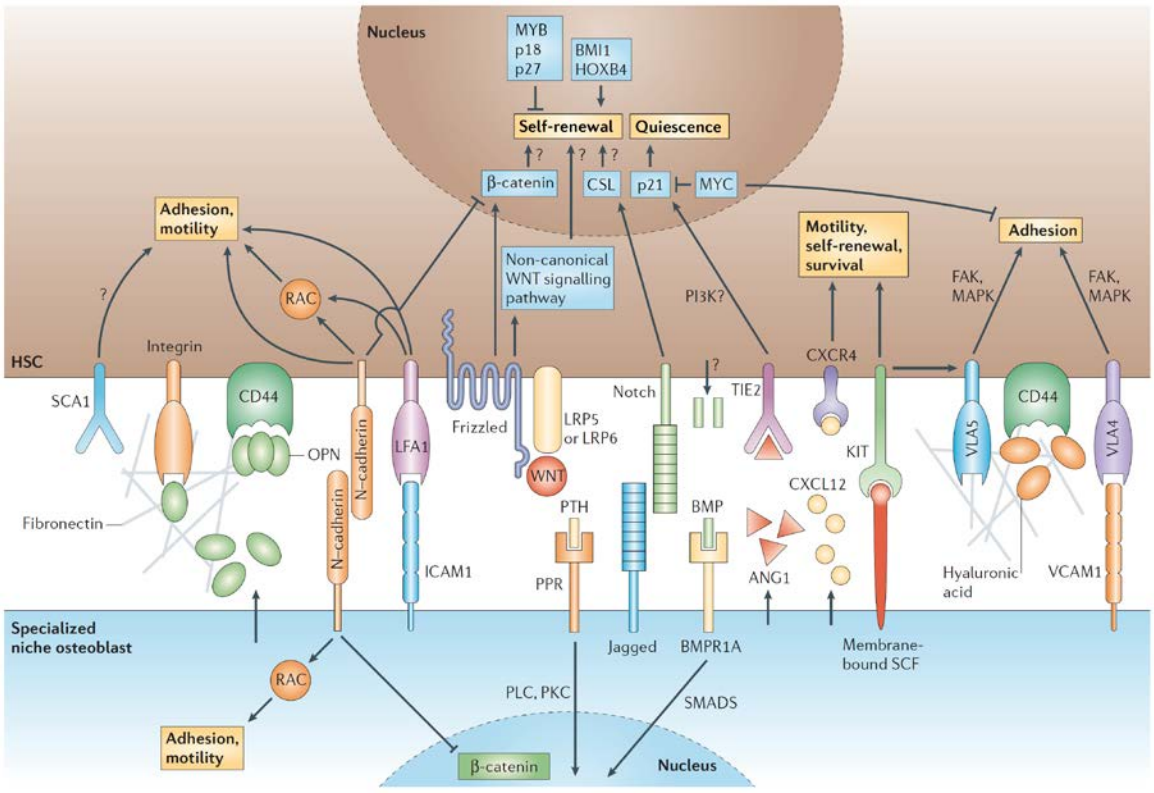

Figure 2

A schematic representation of the signals constituting the stem cell- niche cross talk in the hematopoietic niche. In addition to soluble and cellular factors here depicted, a vatse array of chemo-physical variables (e.g. elasticity of the surrounding matrix, dissolved

oxygen, concerted signalling with other cell types) is involved in controlling and preserving the stem cell pool. (adapted by A. Wilson and A. Trumpp, Nat. Rev. Immun.2006)

Finally, the advent of biomaterials allowed challenging the hypothesis that cell cultures were oversimplified by comparing traditional techniques with approaches that could allow in vitro the a more physiological resemblance of the original tissues. Remarkably, scientific literature keeps on increasing the amount of data showing how the introduction of the third dimension by means of different techniques or biomaterials has a dramatic influence on many aspects of cultured cells. For example, gene and protein expression, differentiation, cytoskeleton organization, proliferation rate, response to stimuli and drugs, and overall functionality, were reported to be remarkably affected by 2D cultures when compared to *in vivo* parameters. However, the use of 3D models seem to be able to at least partially rescue the physiological functionality of the cells.

In parallel with an increase of the availability of biomaterials, many supporting platforms started to be developed in order to maximize the exploitation of the 3D culture systems, particularly bioreactors for 3D cell cultures. Bioreactors can be intended as "devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature, dissolved oxygen, defined recirculation of medium)" [11,13]. The use of bioreactors is instrumental to overcome some of the challenges posed by 3D cultures. For example, cell seeding in a porous structure can be performed manually, but the distribution of the cells results as non-homogeneous and lacks reproducibility; on the contrary, the use of means to control the relative motion between a cell

suspension and a scaffold will result in a more standardized process. Another parameter that makes bioreactors of crucial importance is the increased mass transport throughout the whole volume of the cell construct. In fact, diffusion of nutrients and waste removal can represent a bottleneck for cell viability towards the core of a cellularized 3D biomaterial. By forcing a relative motion of the medium through the construct, bioreactors can instead decrease the limits of the maximum size of the cultured construct. Finally, the possibility to administer active mechanical stimuli to the construct can promote the activation of tissue specific pathways that would otherwise remain silent in monolayer culture.

Direct perfusion bioreactors, in which the culture medium is forced in a controlled manner to pass through the porous structure of the material, are an excellent example of devices for research use and clinical applications. In fact, being relatively easy to use and suitable to carry out cell culture with basically any type of scaffold, they represent a convenient tool for research; in addition, due to the specific advantages that they offer in terms of reproducibility and scalability, perfusion bioreactors can satisfy the technical requirements of tissue engineers to move from the bench to the bedside for regenerative medicine applications.

Aim of the thesis

Currently, the majority of current cultures is still carried out with established techniques like the exploitation of 2D supports, the use of tissue-derived immortalized cell lines, and the administration of un-physiological doses of soluble factors to induce a biological response. However, the lack of structural and physical cues often leads to biological artifacts, from the total loss of cellular function to the lack of correlation

between the predicted and actual results when the experimental model shifts from in vitro to in vivo.

Hence, in this work I test the hypothesis that recapitulating in vitro chemo-physical components of the native cell environment can uniquely maintain the original function and the phenotype of cultured cells. Therefore, the critical aspects are (i) the choice of a suitable source of cells, and (ii) the engineering of the culture conditions. In first instance, it is proposed that freshly isolated adult cells, as opposed to cell lines, are needed to mimic physiological and pathological processes occurring in animal tissues and organs. Secondly, in vitro culture conditions need to be adapted to support cell viability, function, and growth. In particular, the proposed approach relies on the combination of the cells with a suitable biomaterial able to provide a 3D environment for cell adhesion and suitable to allow complex spatial interactions with neighboring cells. The concept of the third dimension as a critical parameter able to influence cell physiology is challenged in different contexts. The complexity of the proposed culture systems, due to the high number of variables among 2D and 3D experimental groups, is such that the precise dissection of the single contributions is not obvious. However, we propose that the combination of a physiological 3D architecture with a suitable biomaterial provide technological and biological advantages able to trigger further investigations.

Notably, the material itself can be chosen so to mimic the native organ, e.g. the mineralized matrix of bone substituted in vitro by a ceramic material. Additionally, we suggest that the use of bioreactors as supportive technologies can exploit the full potential of 3D cell cultures.

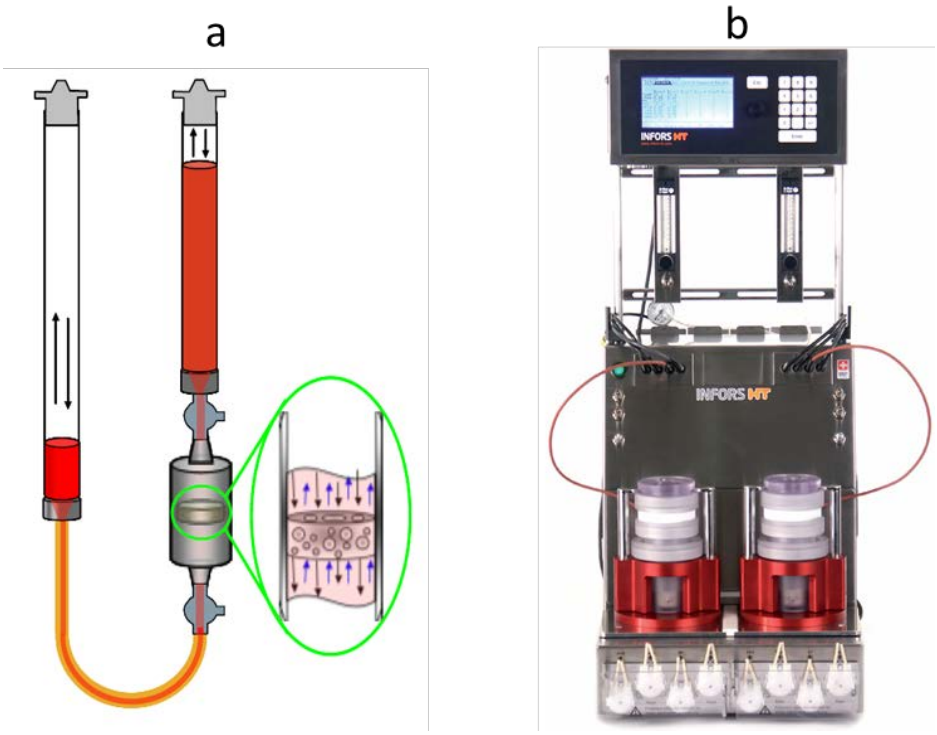

Figure 3

Two examples of perfusion bioreactor for cell cultures. The first bioreactor is a simple tubing system that allows alternate perfusion of medium through the scaffold, (magnified in the green oval) (a). A more sophisticated bioreactor system, developed with the perspective of fully automating and standardizing cell culture, with the capacity of enabling monitoring and control over chemo-physical culture parameters (b).

Despite implying an increase in the complexity of the procedures required to execute experiments based on 3D cell cultures, it is proposed that the relevance of the results surpasses the efforts required to implement new culture models.

Experimental work

In the first chapter of my thesis, I focused on the validation of a platform for the expansion of bone-marrow derived stromal cells (MSC). This heterogeneous population of adherent cells is characterized by a certain array of markers, clonogenic

potential, and multipotency, i.e. the ability to differentiate into different stromal tissues. These distinctive features are either impaired or lost with the progression of the culture on plastic. Here we demonstrate that conventional expansion in monolayer on plastic dishes (2D) can be entirely bypassed by culturing freshly isolated progenitor cells within the pores of 3D scaffolds in a perfusion-based bioreactor system.

Cells cultured for the same amount of time or for the same amount of doublings were then analyzed in terms of maintenance of clonogenic capacity and differentiation potential. In addition, microarray analysis was performed on 5 donors with 2D and 3D cultured cells to investigate the regulation of functional gene clusters.

As a result, the bioreactor-based platform was validated not only as a streamlined approach to expand MSC that maintain at a higher extent progenitor features, but also as a valuable tool to recreate in vitro an engineered stromal niche.

In the second chapter of the thesis the focus was moved to exploit the unique features of 3D cultures on the recapitulation of the thymic stroma in vitro. Thymic stroma is mainly composed by thymic epithelial cells (TEC) that constitute a unique 3D epithelial structure. Freshly isolated TEC cultures from adult mice are currently inadequate to represent the physiology of the thymus due to the loss of function that TEC undergo soon after explant. However, the thymus is an extremely plastic organ with high cell turnover rate, so reasoning that TEC have an intrinsic capacity to proliferate, it was hypothesized that their impairment in traditional cultures could be prevented by providing an engineered 3D environment. Thus, this chapter describes the evolution of a culture system able to manufacture in vitro a thymic organoid constituted by functional TEC that can suits as a model to investigate thymus physiology and, prospectively, engineer “thymus transplants” for clinical applications.

Finally, in the third chapter of the thesis, the concept of 3D stromal tissue engineering is applied to the hematopoietic niche, a specialized microenvironment devoted to regulate hematopoietic stem cells (HSC) quiescence and activity through a wide array of chemo-physical cues. Due to the strategic clinical relevance of HSC, many laboratories explore HSC biology through in vivo models, with all the limitations related to the translation to human clinical practice, or through in vitro models, often rudimental when compared to the complexity of a multicellular, extracellular matrix-embedded environment like the stem cell niche. Only recently, innovative approaches were proposed to recapitulate some aspects of the niche and to dissect the extrinsic factors of the HSC microenvironment to gain insight stem cell function and the mechanisms that control their diverse fates. However, the approach of this thesis is focused on the recapitulation of the complexity of the niche, where multiple cell types like MSC, endothelial, and perivascular cells all play in concert to regulate the chemo-physical cues controlling HSC metabolism. Starting from previous reports in which freshly harvested bone marrow- or adipose tissue-derived cells can be cultured within porous scaffolds, allowing the formation of an organized 3D stromal tissue, we propose that cellularized constructs can be cultured in perfusion bioreactors to reconstruct the HSC niche through the controlled modulation of several parameters.

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CHAPTER I

Expansion of human mesenchymal stromal cells from fresh bone marrow in a 3D scaffold-based system under direct perfusion

*Adam Papadimitropoulos[1], *Elia Piccinini[1], Sophie Brachat[2], Alessandra Braccini[1], David Wendt[1], Andrea Barbero[1], Carsten Jacobi[2] and Ivan Martin[1]

1. Departments of Surgery and of Biomedicine, Institute for Surgical Research and Hospital Management, University Hospital Basel, University of Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland

2. MusculoSkeletal Diseases, Novartis Institutes for Biomedical Research, Basel, Switzerland.

* = Equally contributing authors

Short title: 3D expansion of MSC under perfusion

Key words: bioreactor, microarray, multipotency, stem cells, regenerative medicine

Abstract

Mesenchymal stromal/stem cell (MSC) expansion in conventional monolayer culture on plastic dishes (2D) leads to progressive loss of functionality and thus challenges fundamental studies on the physiology of skeletal progenitors, as well as translational applications for cellular therapy and molecular medicine. Here we demonstrate that 2D MSC expansion can be entirely bypassed by culturing freshly isolated bone marrow nucleated cells within 3D porous scaffolds in a perfusion-based bioreactor system. The 3D-perfusion system generated a stromal tissue that could be enzymatically treated to yield CD45- MSC. As compared to 2D-expanded MSC (control), those derived from 3D-perfusion culture after the same time (3 weeks) or a similar extent of proliferation (7-8 doublings) better maintained their progenitor properties, as assessed by a 4.3-fold higher clonogenicity and the superior differentiation capacity towards all typical mesenchymal lineages. Transcriptomic analysis of MSC from 5 donors validated the robustness of the process and indicated a reduced inter-donor variability and a significant upregulation of multipotency-related gene clusters following 3D-perfusion- as compared to 2D-expansion. Interestingly, the differences in functionality and transcriptomics between MSC expanded in 2D or under 3D-perfusion were only partially captured by cytofluorimetric analysis using conventional surface markers. The described system offers a multidisciplinary approach to study how factors of a 3D engineered niche regulate MSC function and, by streamlining conventional labor-intensive processes, is prone to automation and scalability within closed bioreactor systems.

Introduction

MSC are receiving an increasing experimental and clinical interest, owing to the large degree of plasticity and the capacity to modulate the immune system or the phenotype of cancer cells [1]. Their use is thus advocated for treatment of various genetic, haematologic or immunologic pathologies and in the emerging field of regenerative medicine [2–4]. For most of these potential applications, given the low frequency among bone marrow nucleated cells (around 0.01%), MSC are typically expanded by sequential passages in monolayer (2D) cultures. However, this process is associated with a progressive reduction of their clonogenicity and multilineage differentiation capacity, and is often accompanied by cellular senescence [5,6].

Studies on different cellular systems have led to the concept that maintenance of 'early progenitor' properties generally requires a tissue-specific microenvironment or niche [7–11], which can hardly be resembled by the plastic substrate and 2D configuration of tissue culture flasks [12]. Various attempts have thus been reported to expand MSC in three-dimensional (3D) environments, based on suspension culture in the presence of dynamic flow [13,14], on microcarrier beads [15–17] or on a rotating bed bioreactor system [18,19]. Despite the promising results obtained, however, these approaches required an initial phase of MSC growth on plastic, which is intrinsically associated with the selection of the adherent cellular fractions, possibly already depleted of the less adherent earlier progenitors [20], and the loss of most hematopoietic lineage cells. Indeed, non-mesenchymal bone marrow cells were proposed to be involved in regulating MSC function [21] and have been demonstrated to enhance growth of MSC with clonogenic properties [22,23].

We previously reported that the continuous perfusion of freshly isolated human bone marrow cells directly through the pores of 3D ceramic-based scaffolds resulted in the

reproducible generation of tissue constructs, which were highly osteogenic upon ectopic implantation in nude mice [24]. By eliminating the 2D culture step, the system not only streamlined the MSC culture process, but also supported the maintenance of hematopoietic lineage cells, including some of the early progenitors (i.e., CFU-GEMM), thereby establishing some features of the bone marrow niche [25].

In this study, we aimed at investigating the use of the above described 3D scaffold-based perfusion system for human MSC expansion. For this purpose, the generated constructs were enzymatically processed and the retrieved cells were phenotypically and functionally compared to those generated following conventional expansion protocols. Furthermore, a microarray analysis was introduced to identify potential new molecular markers and pathways differentially regulated as well as to validate the robustness of the process across different donor preparations.

Materials and methods

Bone Marrow Aspirates

Bone marrow aspirates (20ml volumes) were obtained from five healthy donors (average age 45 y.o.) after informed consent during orthopaedic surgical procedures in accordance with the local ethical committee (University Hospital Basel; Prof. Dr. Kummer; approval date 26/03/2007 Ref Number 78/07). Nucleated cells were isolated from aspirates by means of red blood cells lyses buffer (pH 7.2) containing 0.15M NH_4CL, 1mM $KHCO_3$ (both from Sigma, Switzerland) and 0.1mM Na_2EDTA (Fluka, Switzerland). The average clonogenicity (number of fibroblast colony-forming units; CFU-f) in the fresh marrow aspirates was 0.008% ± 0.002%.

Culture Medium

Unless otherwise stated, complete medium (CM) consisted of α-Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), 10mM HEPES buffer, 1mM

sodium pyruvate, 10000U/ml penicillin and 10000µg/ml streptomycin (all from GIBCO, Switzerland). CM was then supplemented with 10 nM dexamethasone and 0.1 mM L-ascorbic acid-2-phosphate (both from Sigma, Switzerland) and with 5ng/ml fibroblast growth factor-2 (FGF-2, R&D systems, Europe),.

MSC Culture

Using a perfusion bioreactor system described in [26] and now commercially available by Cellec Biotek AG (http://www.cellecbiotek.com), an average of 66×10^6 freshly isolated bone marrow–nucleated cells were perfused for 5 days through 8-mm-diameter, 4-mm-thick disks of porous (total porosity, 83% ± 3%; accessible surface area 3200 cm^2) hydroxyapatite ceramic (Engipore; Fin-Ceramica Faenza, Faenza, Italy, http://www.finceramicafaenza.com) at a superficial velocity of 400 µm per second. After 5 days, culture medium was replaced and perfusion culture was performed at a velocity of 100 µm per second for additional 14 days and changing the medium twice per week. In order to establish a comparison with the standard culture process, MSC expansion in 2D (in 56 cm^2 Petri dishes; BD Biosciences) was performed for up to 19 days without passaging using similar initial cell numbers/surface area and schedule of medium changes, as in the 3D expansion condition.

Cell Extraction

At the end of the expansion phase in the 3D culture system, cells were extracted by substituting the CM with a solution of 0.3% collagenase (collagenase) and perfusing the ceramic constructs for 40 min followed by 0.05% trypsin/0.53 mM EDTA solution (trypsin) for additional 15 min both at 400 µm per second. Extracted cells were subsequently sorted using anti-CD45-coated magnetic beads (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. 2D-expanded cells were retrieved by using the same enzymatic solutions, i.e. collagenase for 40 min and trypsin for 5 min.

The fraction of dead cells, preliminarily assessed by assessed by Trypan blue exclusion (Sigma, Switzerland), was negligible (less than 3%), with no obvious differences between the experimental groups. Both $CD45^+$ and $CD45^-$ viable cell populations were assessed for the ability to form fibroblastic colonies. The $CD45^-$ populations were further characterized by flow cytometry, gene expression by means of microarray analysis and quantitative real-time (QRT) PCR or tested for the multilineage differentiation capacity, as described below.

Clonogenicity (CFU-f) and flowcytometry assays

CFU-f assays (n=5) of bone marrow or expanded cells were performed by plating 4400 freshly isolated mononucleated cells or 4 expanded cells per cm^2 in tissue culture dishes, respectively. The procedure was optimized following preliminary experiments with serial dilutions of plated cells. After 14 days of culture, cells were fixed in 4% formalin, stained with 1% methylene blue and the number of colonies was counted.

2D or 3D-perfusion expanded $CD45^-$ cells from one donor were incubated with antibodies against CD29, CD31, CD34, CD44, CD45, CD49a, CD73, CD90, CD105, CD117, CD133, CD144, CD146, CD166, CD271, Alkaline phosphatase, SSEA-1 or human leucocyte antigen (HLA)-DR (all from BD Biosciences). Isotype IgGs were used as controls (all from BD Biosciences). After washing, cells were resuspended in FACS buffer (0.5% human serum albumin, 0.5 mm EDTA in PBS) and analysed with a FACSCalibur flow cytometer (BD Biosciences).

RNA Extraction and Microarray analysis

Total cellular RNA (40ug) was extracted from 2D or 3D-perfusion expanded CD45- cells, obtained from 5 independent experiments/donors, using RNeasy Micro kit (Qiagen, Valencia, CA) following the protocol supplied by the manufacturer. RNA were hybridized to Affymetrix Human HG-U133plus2 GeneChip arrays according to the

manufacturer recommendations. All the data have been deposited in the Gene Expression Omnibus database with experiment series number GSE52896 available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52896.

Arrays pre-processing and analysis were performed using R and the Bioconductor package (http://www.bioconductor.org/) and passed through array quality control using the AffyQCreport tool. Raw intensities were normalized using RMA and scaled to a 2% trimmed mean of 150. Probes with normalized expression values below 50 in both groups were filtered out. Differential gene expression was performed using Limma. Probes were annotated using the platform annotation file version 31 from NetAffx. Genes with a fold change higher than 2 and an adjusted P-value below 0.05 (Benjamini and Hochberg multiple testing correction) were considered regulated. Data from microarrays were analysed by Principal Component Analysis (PCA) using TM4 Multi Experiment Viewer (MeV), available at http://www.tigr.org/software/tm4/mev.html in order to ascribe the overall variability of the sample to a limited number of variables.

To validate microarray data, the expression of a set of genes was evaluated by quantitative real-time (QRT) PCR (Supplementary Figure 2). Total RNA extraction, cDNA synthesis and real-time reverse transcriptase-polymerase chain reaction (RT-PCR; 7300 AB Applied Biosystem) were performed to quantitate expression levels of the following genes of interest: CXCL12 (CXCL12-Applied Biosystems, Ref. Number: Hs00171022_m1), STC1 (STC1- Applied Biosystems, Ref. Number: Hs00174970_m1), EDNRB (EDNRB-Applied Biosystems, Ref. Number: Hs00240747_m1), FZD5 (FZD5-Applied Biosystems, Ref. Number: Hs00258278_s1), CXCL5 (CXCL5-Applied Biosystems, Ref. Number: Hs01099660_g1), KYNU (KYNU-Applied Biosystems, Ref. Number: Hs01114099_m1), CCL20 (CCL20-Applied Biosystems, Ref. Number:

Hs01011368_m1), TAC1 (TAC1-Applied Biosystems, Ref. Number: Hs00243225_m1), DNER (DNER-Applied Biosystems, Ref. Number: Hs01039911_m1), EREG (EREG-Applied Biosystems, Ref. Number: Hs00914313_m1), NR4A3 (NR4A3-Applied Biosystems, Ref. Number: Hs00545009_g1), SLC6A15 (SLC6A15-Applied Biosystems, Ref. Number: Hs00375196_m1) and SNF1LK (SNF1LK-Applied Biosystems, Ref. Number: Hs00545020_m1). 18s was used as housekeeping (18s-Applied Biosystems, Ref. Number: Hs03003631_g1)

Bionformatic Analysis

Gene Set Enrichment Analysis (GSEA)

The list of regulated genes was ranked according to the relative fold-change and loaded in GSEA software (http://www.broadinstitute.org/gsea/index.jsp; ver. 2.0.12). A variety of genesets from the Molecular Signatures Database (MSigDB) were analyzed (http://www.pnas.org/cgi/content/abstract/102/43/15545). The list of genes related to osteogenic differentiation was based on the Human Osteogenesis RT^2 Profiler™ PCR Array (SABiosciences).

Database for Annotation, Visualization and Integrated Discovery (DAVID) and Cytoscape Functional enrichment analysis for up- and down-regulated genes (2 fold with an adjusted pvalue below 10^{-2}) was performed using the open-source web-based DAVID platform (http://david.abcc.ncifcrf.gov/) including Gene Ontology (GO) and Pathways categories. Enriched functional categories and pathways were clustered by gene overlap using Enrichment Map in Cytoscape [27,28] and labelled for recurrent keywords using the WordCloud plugin (http://baderlab.org/Software/WordCloudPlugin). In the generated Cytoscape

diagram, the node size is proportional to the number of genes defining the node. Edges connect nodes that share common genes and edge thickness is proportional to the number of shared genes between nodes.

Multilineage differentiation assays

The osteogenic differentiation capacity was tested by culturing cells, obtained from 3 independent experiments/donors, for 2 weeks in CM further supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate. After 2 weeks, cell layers were either stained with alizarin red solution to evidence mineral deposition or assessed for ALP activity normalized to cell numbers, as previously described [29]. Shorter culture time with respect to the commonly used in literature 3 weeks protocol was chosen in order to maximize the differences regarding the *in vitro* osteogenic differentiation capacity of MSC between the two experimental conditions.

The adipogenic differentiation capacity was tested by alternating cycles of cell culture with different media, including 10 µg/ml insulin, 10 µM dexamethasone, 100 µM indomethacin, and 500 µM 3-isobutyl-1-methyl xanthine (adipogenic induction medium) or 10 µg/ml insulin (adipogenic maintenance medium) as previously described [30]. After a total of 14 days, the presence of adipocytes was microscopically documented and quantified following Oil red-O staining.

The chondrogenic differentiation capacity was tested by culturing cells in spherical pellets, formed by gentle centrifugation in 1.5 ml conical polypropylene tubes (Sarstedt, Numbrecht, Germany), in serum-free D-MEM medium (GIBCO, Switzerland) containing

ITS+1 (10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium, 0.5 mg/ml bovine serum albumin, 4.7 µg/ml linoleic acid; Sigma, Switzerland), 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/ml human serum albumin, 100 nM dexamethasone (Sigma, Switzerland), and 10 ng/ml TGF-β1 (R&D Systems, Europe), with medium changed twice weekly. After 3 weeks' culture, pellets were processed biochemically for glycosaminoglycan (GAG) and DNA content and histologically for Safranin-O staining.

Immunosuppression assay

The proliferation of CD4+ T cells, sorted from PBMCs of a healthy donor, in the presence of MSC was performed in 96-well plates following a method described [31]. Briefly, 2D- and 3D-perfusion expanded MSC were seeded at densities of 1250, 5000 and 20000 cells per well and allowed to attach at least 4h at 37oC with RPMI1640 medium supplemented with 10% FBS, 10mM HEPES buffer, 1mM sodium pyruvate, 10000U/ml penicillin and 10000 □g/ ml strept

100000 CD4+ cells in the presence of 1ug/ml of the mitogen phytohemagglutinin (PHA; Remel Europe Ltd. Clipper Boulevard West, Crossways Dartford,Kent, DA26PT UK). After 56h of co-culture, 1 µCi/well ^{3}H-thymidine (GE Healthcare, Little Chalfont, United Kingdom) was added to each well and incubated for additional 16h. Cells were then harvested and the ^{3}H cpm counted by a scintillation beta-counter to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division. Each condition was tested in triplicate.

Statistical analysis

For the microarray analysis, genes with a fold change higher than 2 and an adjusted P-value below 0.05 (Benjamini and Hochberg multiple testing correction) were considered regulated. Results are reported as mean ± SD. Statistical analysis was performed with GraphPad Prism 4.0 (Graph Pad software, La Jolla, CA, USA).

Differences were assessed using Mann–Whitney U-tests and considered statistically significant with $P< 0.05$.

Results

3D-perfusion expansion of freshly isolated MSC

Using a bioreactor system as described in [26] and graphically illustrated in Figure 1, total BM cells were perfused through the scaffold pores for 5 days (cell seeding phase), followed by perfusion of culture medium for further 14 days (cell culture phase). Based on the retrospectively calculated density of CFU-f from the five donors (0.08% ± 0.02%) and assuming that all CFU-f attached to the ceramic scaffolds, an estimated average of $5.4x10^3$MSC were perfused through each scaffold, corresponding to 1.6 MSC per cm^2 of ceramic surface area. This process resulted in the formation of stromal-like tissue structures, including cells of heterogeneous morphologies in physical contact with each other (Figure 2a). Instead, conventional cell culture in Petri dishes using similar cell density per surface area led to the generation of adherent cells, typical of the fibroblastic phenotype (Figure 2b). Enzymatic retrieval of the cells from both conditions and labelling for CD45 indicated the presence of a significantly higher percentage of cells of the hematopoietic lineage after expansion in 3D-perfusion as compared to 2D (19.3 ± 5.7 % vs 6.0 ± 4.5 % CD45+) (Figure 2c). The extent of MSC proliferation in the 3D perfusion system, assuming that all harvested CD45- cells (total of 1.36 +/- 0.34 × 10^6 cells/scaffold) were of the mesenchymal lineage and derived from the initial relative number of seeded CFU-f, retrospectively estimated by clonogenicity assays, was of 7.6 ± 1.7 doublings, corresponding to about 0.4 doublings/day. MSC growth in plastic dishes within the same time frame of 19 days (total of 1.76 +/- 0.46 × 10^6 cells/dish) was significantly higher, corresponding to 0.74 doublings/day (Figure 2d). Based on the measured cell yields, the same numbers of

cells occupying 56 cm^2 of a 10cm diameter Petri dish could be expanded in ~0.2 cm^3 of scaffold volume.

Phenotypic characterisation of 3D-perfusion expanded MSC

In order to investigate the phenotype of the mesenchymal cells, retrieved cells were negatively sorted for the expression of CD45. Results displayed in Figure 3 indicate a large overlap in the cytofluorimetric profile of the two cell-expanded groups, without clear-cut differences in the presence or absence of specific cell populations. However, as compared to 2D-expansion culture, a lower percentage of MSC expanded by 3D-perfusion expressed CD90 78.2% vs 99.8%), CD105 (61.2% vs 98.9%), CD166 (87.1 vs 99%) and ALP (5.8% vs 18.5%), a marker associated with the osteoblastic differentiation of MSC. Moreover, slightly higher percentage of 3D-perfusion expanded MSC were positive for HLA-DR (22.8% vs 10.8%) or for CD146 (25.2% vs 11.6%) and SSEA-1 (11.4% vs 7.6%), which were proposed to be associated with progenitor cell properties [32–37].

Microarray analysis of 2D- and 3D-perfusion expanded MSC

In order to broaden the search of potential differentially expressed markers and to validate the robustness of the process across different donor preparations, the CD45-fractions of bone marrow cells expanded by 3D-perfusion or in 2D from 5 independent donors were profiled using expression microarrays. Exploratory analysis using PCA (data dimensionality reduction) was performed in order to reveal correlations between the samples [38]. By dot-plotting the data derived by the two experimental groups, it is possible to estimate the similarity between each sample as a function of the distance of each pair of dots. This analysis shows a striking separation of the samples from 2D and 3D-perfusion on Principal Component 1 (PC1), confirming that

culture conditions represented the most influential factor in discerning among cell preparations (Figure 4a). Interestingly, samples derived from cells cultured in 2D were more spread along the PC2 axis as compared to 3D-perfusion ones, suggesting a higher inter-donor variability induced by 2D-expansion. After pre-processing, we identified 702 genes (343 up-regulated and 359 down regulated) with a fold change of 2 and an adjusted p-value of 10^{-2}. A list of the 10 more up- and down-regulated genes is reported in Table 1.

In order to investigate the pathways associated with the 2D versus the 3D cultures, we performed a Gene Ontology (GO) enrichment and cluster analysis using the online web-platform DAVID on the derived list of regulated genes. The statistically enriched pathways can be visualized as an enrichment map with nodes being pathways and edges representing the overlap in genes in these pathways (Figure 4c). The main GO categories increased in 3D-perfusion vs 2D-expanded MSC were the "Monosaccharides metabolic processes (fructose and glucose)", "Chemokine activity", "Inflammatory response", "Response to hypoxia" and "Negative regulation of apoptosis" (Supplementary Table 1). Consistent with the multicellular tissue-like morphology observed during 3D-perfusion expansion, the GO functional categories "Positive regulation of multicellular organismal process" and "Extracellular space" were also significantly over-represented in the list of up-regulated genes in 3D culture. Conversely, GO categories related to "Fat-related (phospholipid and sphingolipid) and organophosphate metabolic processes" as well as to cytoskeleton, contraction and adhesion were found to be decreased in the 3D-perfusion vs 2D-expanded MSC (Supplementary Table 2). Figure 4b displays a representation of the resulting GO categories and the identified pathways from Supplementary Tables 1 and 2, linked to the underlying biological processes. Interestingly, "Bone development" was found to

be up-regulated in the 2D-expanded cells and appeared to be consistent with the increased protein expression of ALP. Furthermore, one of the most significant 3D up-regulated geneset uncovered using GSEA analysis is the PluriNet [39], a matrix of global gene expression profiles of various types of stem cells, supporting the more "stem cell" like transcriptional footprint of our 3D-perfusion model. Also the geneset describing osteoblastic differentiation was down-regulated in the 3D-perfusion vs the 2D-expanded MSC (Figure 4c).

Validation of the in vitro functionality of 3D-perfusion expanded MSC

We next investigated whether the differential gene expression accounting for multi-potency maintenance and differentiation was mirrored in the functionality of CD45- cells expanded by 3D-perfusion or 2D. The CD45- fraction of the 3D perfusion-cultured cells included a 4.3-fold higher percentage of clonogenic cells (Figure 5a) than that of cells expanded on plastic for the same time (respectively 17% vs 4%), suggesting a better preservation of progenitor cell features. This hypothesis was further confirmed by a more efficient multi-lineage differentiation capacity upon exposure to typical chondrogenic, osteogenic and adipogenic conditions, as determined by histochemical and quantitative biochemical assays (Figure 5b). Since the number of doublings by MSC expanded for 19 days under 3D-perfusion or 2D was different, cell populations were compared also using a shorter culture time in 2D (i.e., 14 days), leading to 9.1 total doublings and thus more similar to the 3D-perfusion group. Both the clonogenic cell fraction and the multilineage differentiation profile of the shorter expansion time in 2D (data not shown) were comparable to those determined for the longer expansion time. Notably, both 2D- and 3D-perfusion expanded MSC cells shared similar anti-proliferating effects on activated CD4+ cells when co-cultured in vitro (Supplementary Figure 1). Lastly and as expected, CD45+ cells from both experimental groups did not

contain adherent fibroblastic clonogenic cells when re-plated in Petri dishes, confirming efficient magnetic depletion (data not shown).

Discussion

We have developed a system for the expansion of MSC which entirely bypasses the use of 2D surfaces by seeding and expanding fresh bone marrow preparations directly within the pores of 3D scaffolds under perfusion flow. As compared to the conventional 2D culture system, MSC expanded under 3D-perfusion (i) preserved better their early progenitor properties, as they maintained a higher clonogenicity and a superior multilineage differentiation capacity, (ii) did not lose their anti-proliferative function, based on a standard in vitro assay typically used to claim 'immunomodulation' properties, and (iii) displayed reduced inter-donor variability and consistent upregulation of multipotency-related pathways, as assessed by transcriptomic analysis.

Identifying a strategy for efficient expansion of MSC preserving their functionality is a critical target towards fundamental mechanistic studies on their biological properties, as well as for their prospective clinical use in the field of tissue engineering and regenerative medicine [12]. Among several hurdles, the absence of phenotypic markers that uniquely identify populations of MSC with specific functions challenges the definition of a quality control during MSC culture [40]. Indeed, surface proteins typically used to characterize MSC [41] were not differentially expressed in cells expanded in 2D or by 3D-perfusion, indicating that they are not suitable to capture functional features related to superior clonogenicity and multilineage differentiation. Only a limited set of markers, including CD146 and SSEA-1, were expressed by a larger percentage of 3D-perfusion expanded MSC, consistent with the proposed association

of those markers to earlier progenitor/stem populations of MSC [34–37]. HLA-DR was found to be expressed in both conditions, likely due to the presence of FGF-2 in the culture medium [42,43]. The relatively higher HLA-DR expression observed in 3D-perfusion condition is consistent with previous observations on the effect of hematopoietic cells on MSC [43] and did not alter the anti-proliferative effects of MSC on T-cells. The broader impact of HLA-DR expression on the immunomodulatory properties of MSC is still subject of debate.

A genome wide comparison demonstrated a clear separation between the transcriptomes of MSC expanded in 2D or 3D-perfusion as evidenced by PCA; moreover, a reduced dispersion of 3D-perfused samples indicates that culture conditions can diminish the inter-donor variability that typically affects 2D cultures. Gene set enrichment analysis further demonstrated that following expansion under 3D-perfusion, MSC up-regulated or maintained a transcriptome profile similar to that of other stem cells, supporting the superior maintenance of the experimentally verified MSC multipotency. In this context, after the expansion phase bone related pathways were found down-regulated in the 3D-perfusion group, further indicating better preservation of an undifferentiated MSC phenotype, against the default progression towards the osteoblastic lineage [44]. Consistently, epidermal growth factor like ligands, which were highly upregulated in the 3D-perfusion dataset, were previously shown to be important for maintenance of osteoprogenitor cells at an undifferentiated stage [45]. Following the differentiation induction phase, 2D-expanded cells, displayed a limited osteogenic profile, despite their apparently more “osteoblastic” phenotype after expansion. Although an in vivo test of osteogenicity was not performed, the in vitro data seem to indicate that the spontaneous tendency to express osteoblastic genes does not necessarily reflect a superior efficiency of functional differentiation.

Amongst the highest differentially upregulated genes found in the 3D-perfusion dataset, several ones coded for toll-like receptors (TLR), interleukins (IL) and other chemokines which are known to be involved in processes of cell migration, tissue homeostasis and repair, as well as in the regulation of immunologic responses. In particular, the higher expression of TLR-2 together with IL-6 and IL-8 may indicate the activation of the receptor by its associated ligands, which has been previously proposed to regulate MSC multipotency [46]. The established 3D transcriptional profiles described here highlighted differential expression of several transmembrane related genes, which may represent a starting point for future studies to define novel markers for the prospective isolation of earlier MSC progenitors.

Some of the categories identified from GO enrichment analysis during 3D-perfusion expansion were related to hypoxia, negative regulation of apoptosis and cell metabolism. Previous studies reported the positive role of hypoxia, a physiological feature of the niche of MSC [47], on the cell maintenance in an undifferentiated state, with metabolic features associated with an extended and more genetically stable lifespan [48]. In the described 3D-perfusion culture system, oxygen gradients and thus hypoxic regions may have occurred as a result of the relatively low rate of fluid flow passing through compact areas of cell-laid ECM. Future studies will have to further explore the role of hypoxia by either using smaller sized scaffolds, thereby enhancing oxygen transport, or by performing 2D cultures in hypoxic conditions.

For 2D expanded cells, cytoskeletal binding, contractile fiber and adherence junction pathways were up-regulated. These biomechanical ECM-induced processes were previously reported to influence cell fate [49,50] and induce osteogenesis of MSC, independently from the culture conditions [51]. Indeed, it has been shown that MSC sense the stiffness of their environment through physical contact and contraction of

ECM proteins, which are deposited according to the rigidity of the underlying material surface [50]. Here, the up-regulation of these processes in 2D expanded MSC may be possibly explained by their continuous exposure to the rigid surface of plastic, in contrast to the 3D-perfusion system, where cells were progressively embedded within ECM (Figure 2), of most likely lower stiffness.

The two experimental conditions for MSC expansion differed in multiple parameters of various nature (e.g., 3D vs 2D configuration, ceramic vs plastic substrate, flow-induced shear vs static environment, maintenance vs loss of hematopoietic cells), which can hardly be de-coupled to establish appropriate controls. Thus, while confirming the influence of a dynamic 3D environment on MSC properties [52], the identification of the mechanisms leading to a more functional population of MSC when expanded under 3D-perfusion is beyond the scope of the present work. It is likely, however, that the 3D structure of the scaffold is instrumental to entrap various cells types, including hematopoietic cells [53], and supports the deposition and presentation of extracellular matrix signals known to positively regulate MSC expansion [54,55]. Based on the recent finding that typical stromal populations can form the niche to earlier, less adherent MSC progenitors which are removed with medium changes [20], it would be tempting to speculate that the stromal cell network generated within the scaffold offers the environmental cues required to support maintenance in culture of the earlier progenitors.

In the present study, a ceramic-based material has been used as a surface for initial adhesion and growth of MSC, in order to mimic some features of the mineralized trabeculae surrounding a marrow stromal tissue. It is likely that the use of materials of different composition, architecture and surface properties would provide different priming signals to marrow cells. Thus, the choice of the scaffold included in the

perfusion chamber could represent a critical parameter of the system and at the same time an additional tool to dissect the role of specific factors in maintenance of MSC features. The 3D culture process critically requires the use of direct perfusion, initially in order to uniformly distribute cells throughout the scaffold pores [26] and later to efficiently nourish the cells down to the scaffold core. Moreover, the induced perfusion would also mimic the physiological role of interstitial fluid flow and associated mechanical shear in the bone environment [56–58]. Our previous study, though with animal derived BMSC and slightly different medium composition, has indicated the effect of continuous flow during culture in maintaining the presence of hematopoietic lineage cells [59]. Therefore, an experimental setup involving 3D cell cultures under static conditions or by perfusing pre-sorted CD45- cells from bone marrow preparations could identify the role of hematopoietic lineage cells in the maintenance of MSC functionality.

Conclusions

In this work we have proposed an unprecedented paradigm for human MSC expansion, which – unlike most so far reported methods – does not rely on plastic adherence to initiate the culture. The described system relies on the in vitro establishment of a 3D stromal environment as a biomimetic niche supporting MSC growth while better preserving their functional properties. The complete elimination of the labor-extensive serial passaging in monolayer and the use of a perfusion-based bioreactor open the perspective of a streamlined, automated and controlled MSC expansion within closed systems, possibly addressing not only cell quality issues but also cost effectiveness and standardization of the manufacturing process for clinical and industrial implementation [58]. From a scientific perspective, the culture method offers the possibility to systematically investigate how different parameters (e.g., scaffold

composition, architecture and functionalization, flow rate) regulate the phenotype, growth and function of the generated cell populations, and could be used as an engineered 3D model of the bone marrow stromal environment to study physiological interactions among multiple cell types. Finally, the approach may be extended to other stem cell systems, of interest in fundamental research, molecular medicine and cellular therapy.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest related to the present study.

Table 1. Top-ten of significant up- and down-regulated expressed genes

Probe Set ID	Gene Descirption	Gene Symbol	Function	Fold Change (3D-perfusi vs 2D)
Upregulated				
214974_x_at	chemokine (C-X-C motif) ligand 5	CXCL5	secreted	69
205239_at	amphiregulin	AREG	both	46
205476_at	chemokine (C-C motif) ligand 20	CCL20	secreted	39
230748_at	solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	SLC16A6	tm	32
226281_at	delta/notch-like EGF repeat containing	DNER	tm	27
206336_at	chemokine (C-X-C motif) ligand 6	CXCL6	secreted	26

	(granulocyte chemotactic protein 2)			
204105_s_at	neuronal cell adhesion molecule	NRCAM	tm	24
206376_at	solute carrier family 6 (neutral amino acid transporter), member 15	SLC6A15	tm	23
211506_s_at	interleukin 8	IL8	secreted	21
205767_at	epiregulin	EREG	both	20
Downregulated				
230204_at	hyaluronan and proteoglycan link protein 1	HAPLN1	secreted	-28
204051_s_at	secreted frizzled-related protein 4	SFRP4	secreted	-18
212328_at	LIM and calponin	LIMCH1		-17

	homology domains 1			
227662_at	synaptopodin 2	SYNPO2		-16
225275_at	EGF-like repeats and discoidin I-like domains 3	EDIL3	secreted	-16
228407_at	signal peptide, CUB domain, EGF-like 3	SCUBE3	secreted	-16
220976_s_at	keratin associated protein 1-1	KRTAP1-1		-15
223315_at	netrin 4	NTN4	both	-14
212327_at	LIM and calponin homology domains 1	LIMCH1		-14
212865_s_at	collagen, type XIV, alpha 1	COL14A1	secreted	-13

Figure Legends

Figure 1. Schematic overview **of the experimental setup.** Bone marrow aspirates were seeded into the 3D perfusion system and in conventional Petri dishes. After culture, cells from both systems were enzymatically retrieved and CD45- sorted cells using magnetic beads were analyzed as described.

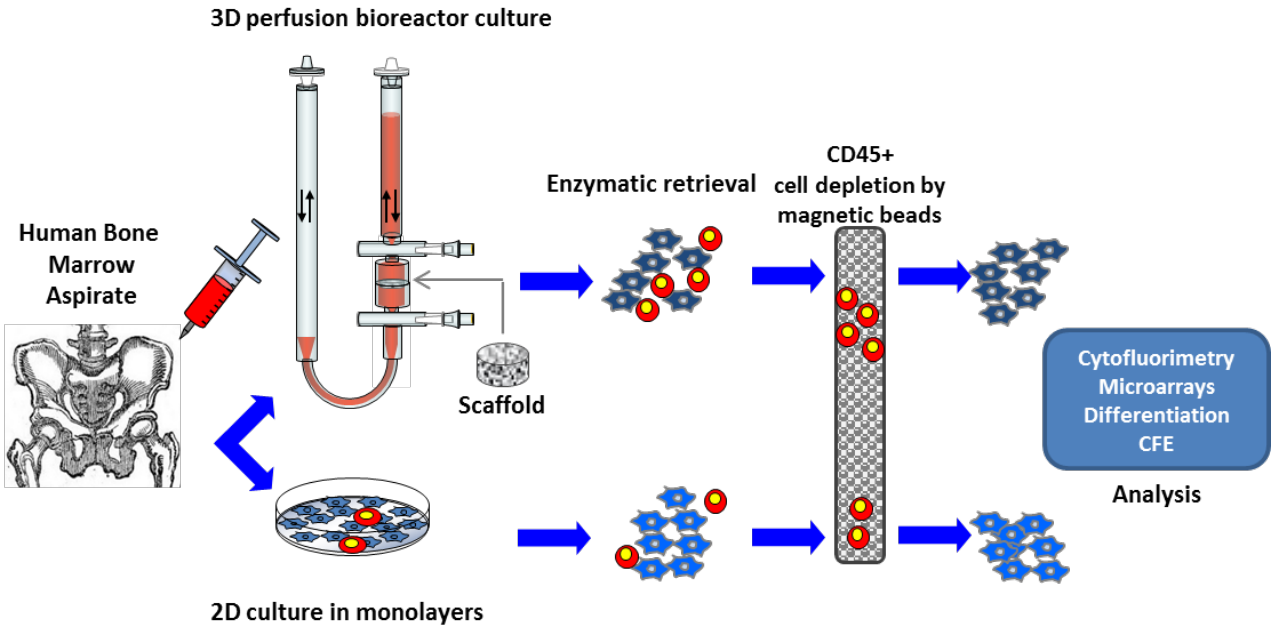


Figure 2. Phenotypical and growth characteristics for 2D and 3D perfused MSC. (a) Scanning electron microscopy imaging of cells within the scaffold display a complex network of branched fibroblastic-like adherent cells and the presence of rounded cells possibly of hematopoietic origin. (b) 2D cultured MSC display a typical flat fibroblastic morphology. (c) Flow cytometry of cultured cells shows a higher frequency of CD45+ cells in the perfusion system. (d) Proliferation rates indicate higher proliferation in 2D as compared to 3D perfusion cultured MSC. Statistically significant differences ($P<0.05$) are indicated with an asterisk (*; n=5).

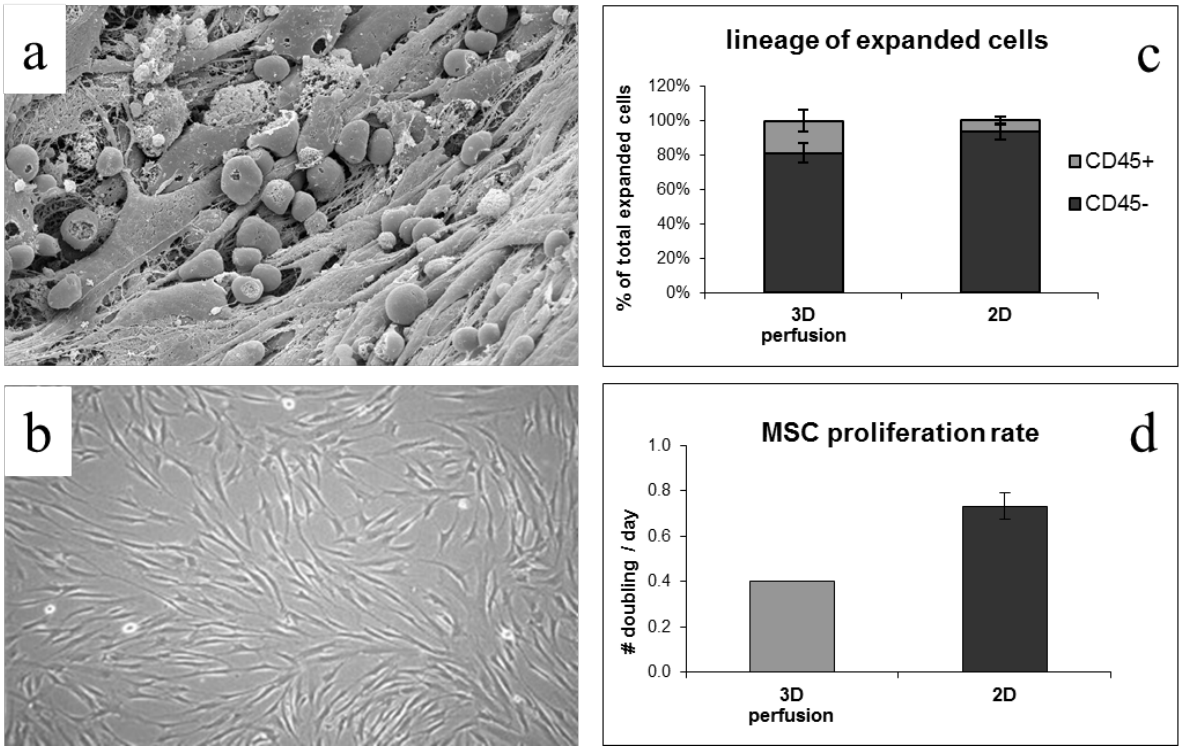


Figure 3. Analysis of the expression of surface markers in 2D and 3D cultured MSC. Colored lines display the frequency of positive cells compared to isotype (gray lines). Most markers were similarly expressed in the two experimental groups. CD90, CD105, CD166, and ALP positive populations were more represented in monolayer culture, while CD146 and SSEA-1 were more represented in 3D-perfusion culture.

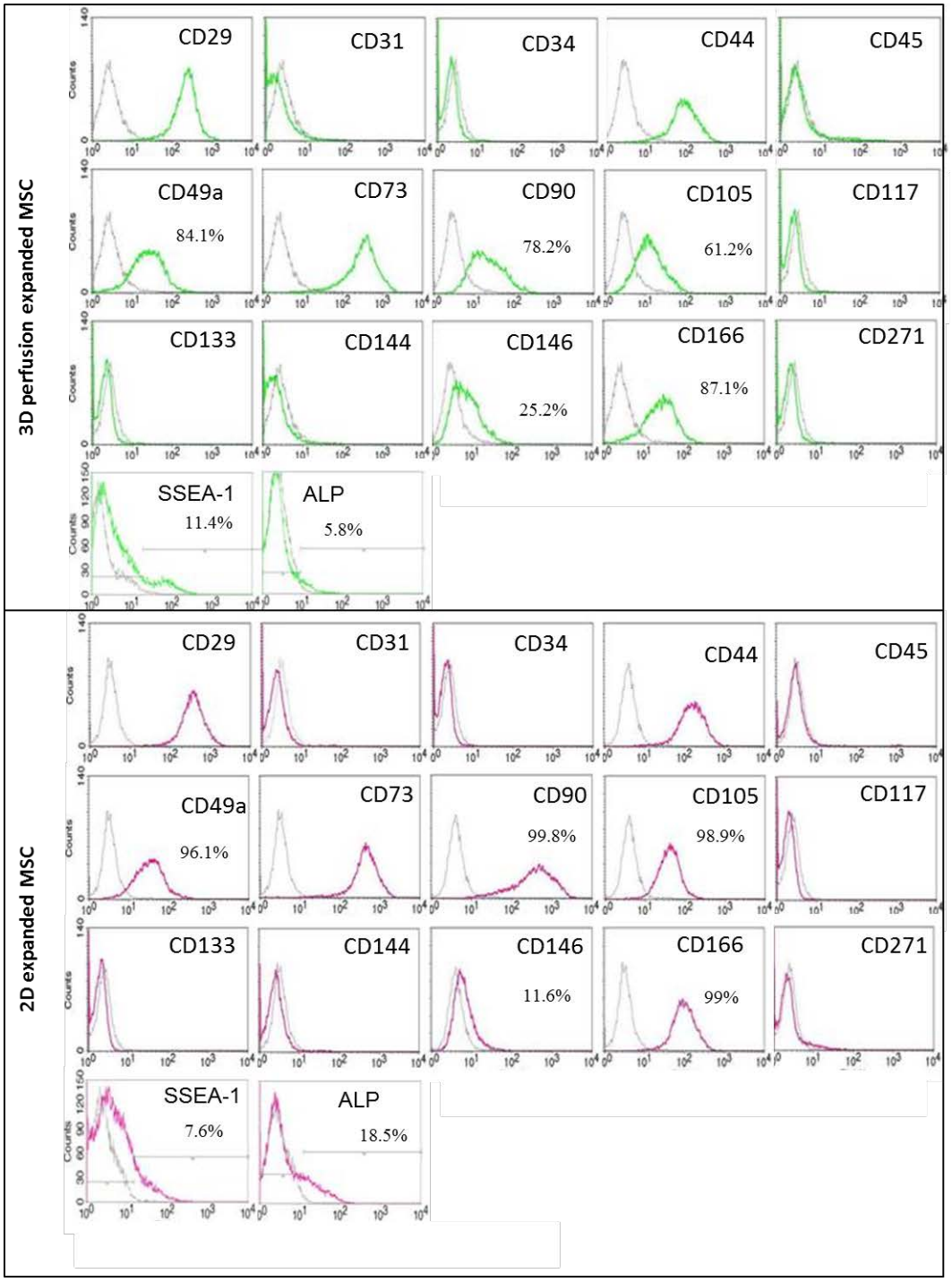


Figure 4. Gene expression analysis of MSC cultured in 2D or 3D perfusion culture. (a) Principal component analysis on global gene expression data. Cells cultured in 3D perfusion system exhibited a significantly different RNA expression profile compared to 2D, with lower inter-donor variability. (b) Cytoscape diagram integrates graphically the most relevant gene ontology biological processes identified by functional annotation (DAVID bioinformatics tool) of regulated genes. Node size (red dots) is proportional to the number of genes defining the node. Edges connect nodes that share common genes in the 2D condition (green edges) or in the 3D perfusion condition (blue edges). Edges thickness is proportional to the number of shared genes between nodes. (c) Gene set enrichment analysis of regulated ranked genes displays that up-regulated genes in 3D perfusion condition are largely overlapping with stem cell related genes (Geneset: Plurinet), while gene related to osteogenic differentiation (Geneset: Osteogenes) are down-regulated. The acronym for NES stands for normalized enrichment score, which is calculated by GSEA software.

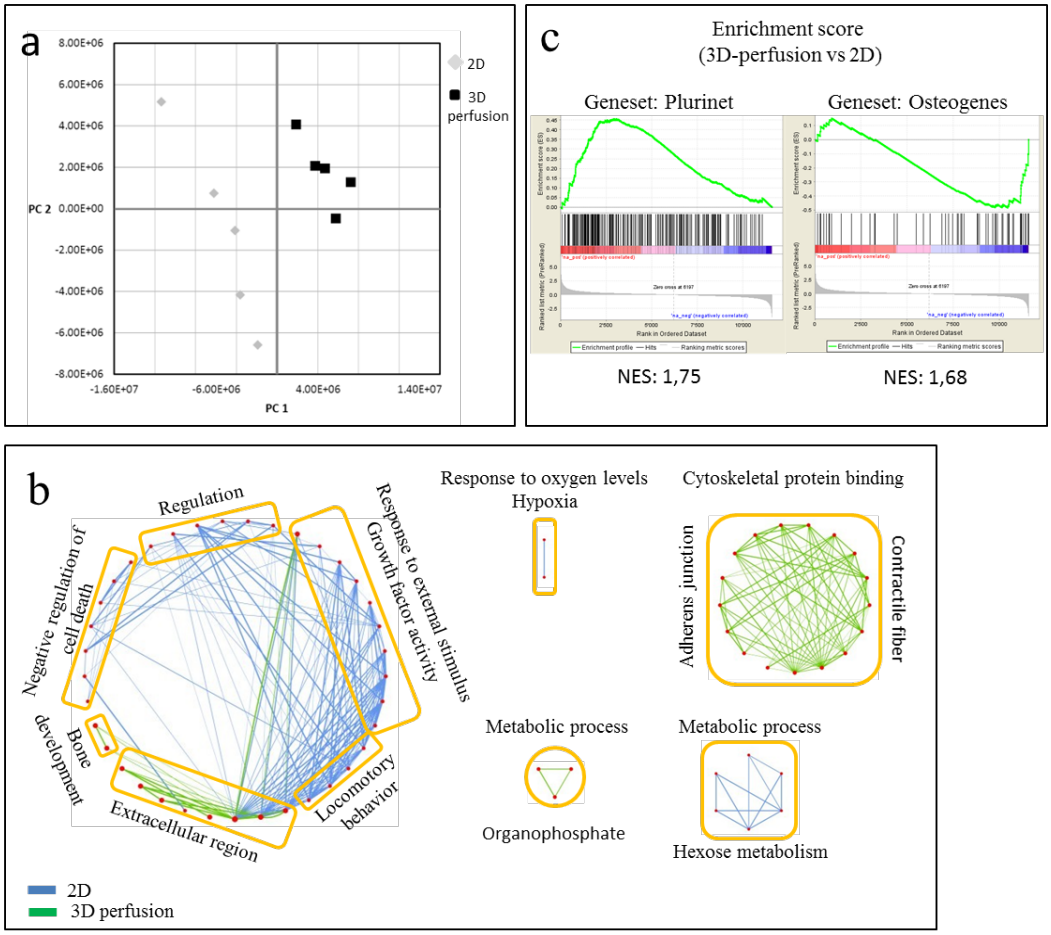
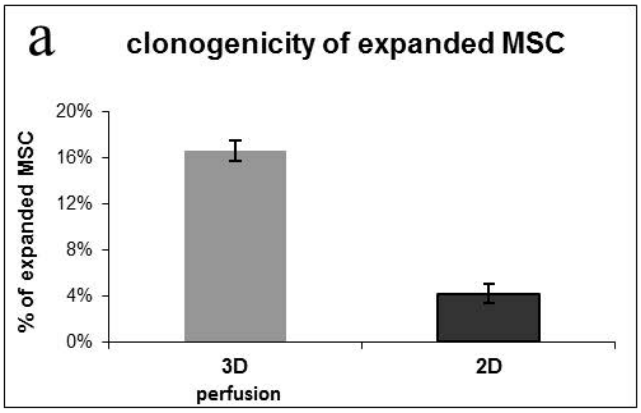
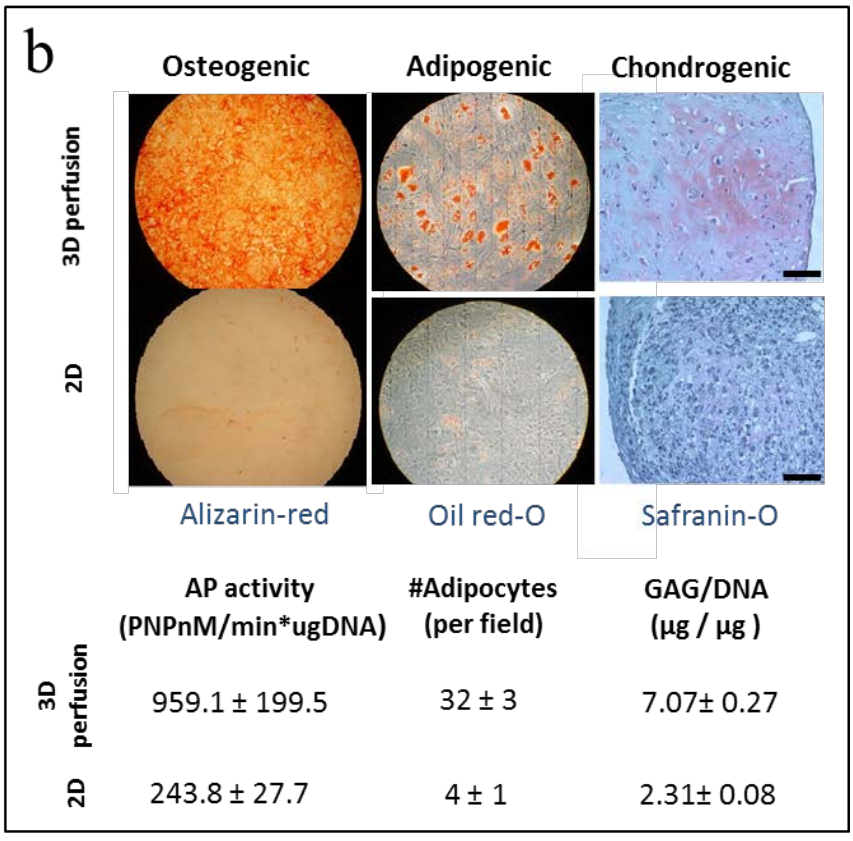


Figure 5. Functional differences between 2D and 3D perfused MSC. Higher (a) frequency of clonogenic cells and (b) differentiation capacity for osteogenic, adipogenic, and chondrogenic lineages with the associated quantifications of 3D perfusion- as compared to 2D-expanded cells. Scale bar: 50um. Statistically significant differences ($P<0.05$) are indicated with an asterisk (*; n=3).





	AP activity (PNPnM/min*ugDNA)	#Adipocytes (per field)	GAG/DNA (µg / µg)
3D perfusion	959.1 ± 199.5	32 ± 3	7.07± 0.27
2D	243.8 ± 27.7	4 ± 1	2.31± 0.08

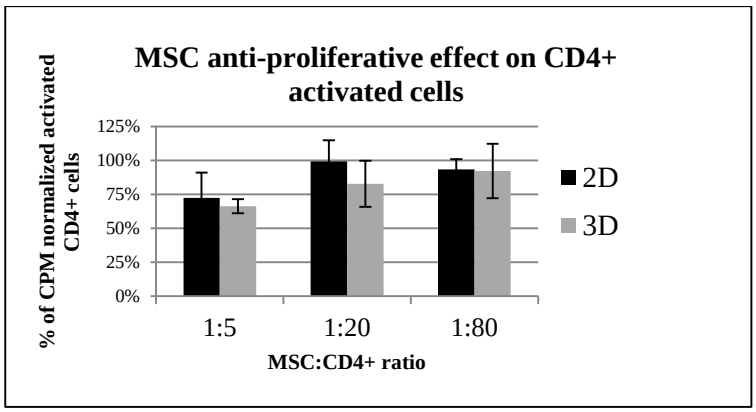
Supplementary Table 1. Biological processes correlated with BMSC genes that are up-regulated of at least two-folds in 3D as compared to 2D culture. Terms are ordered according to their p-values.

TERM	Input genes in GO terms (%)	P-Value
hexose metabolic process	7,0	2,4E-8
monosaccharide metabolic process	7,0	1,8E-7
extracellular space	11,6	6,0E-7
cytokine activity	6,2	6,1E-7
glucose metabolic process	5,4	2,7E-6
extracellular region part	3,3	2,7E-6
chemokine activity	2,5	3,9E-6
fructose metabolic process	3,3	4,4E-6
chemokine receptor binding	13,2	6,4E-6
taxis	5,0	2,1E-5
chemotaxis	5,0	2,1E-5
regulation of cell proliferation	5,4	2,7E-4
magnesium ion binding	7,4	2,8E-4
positive regulation of multicellular organismal process	10,7	2,8E-4
inflammatory response	6,2	3,0E-4
positive regulation of cytokine production	3,3	3,8E-4
Fructose and mannose metabolism	2,5	6,3E-4
Cytokine-cytokine receptor interaction	5,4	6,5E-4
response to wounding	6,2	7,6E-4
locomotory behavior	5,0	7,6E-4
response to organic substance	2,9	7,9E-4
positive regulation of response to stimulus	7,9	8,1E-4
cytosol	3,7	9,8E-4
positive regulation of defense response	9,5	1,1E-3
response to hypoxia	3,7	1,3E-3
response to oxygen levels	2,1	1,3E-3
behavior	8,3	1,3E-3
defense response	7,0	1,5E-3
NOD-like receptor signaling pathway	13,6	1,6E-3
negative regulation of apoptosis	2,9	1,7E-3

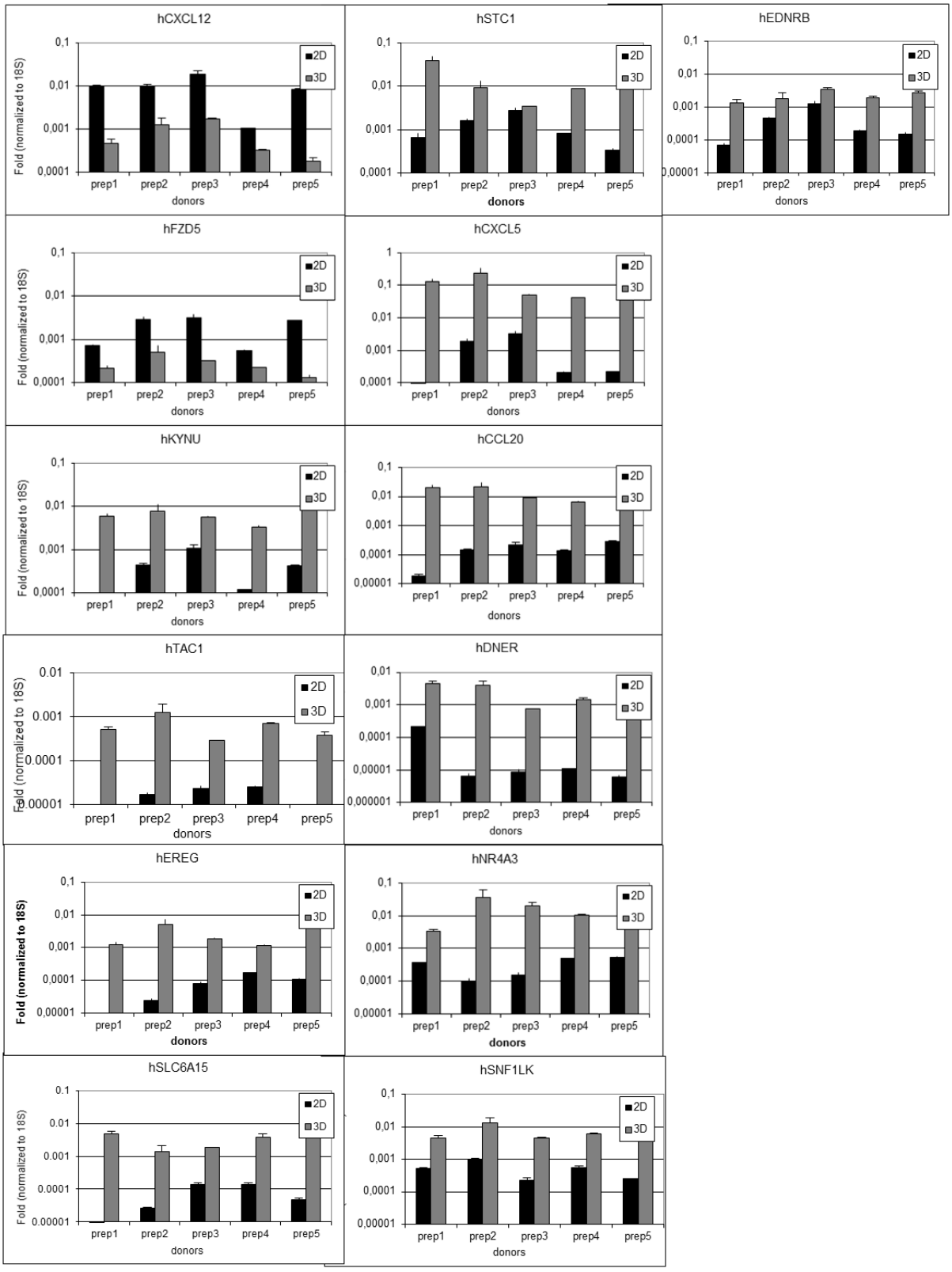
Supplementary Table 2. Biological processes correlated with BMSC genes that are down-regulated of at least two-folds in 3D as compared to 2D culture. Terms are ordered according to their P values.

TERM	Input genes in GO terms (%)	P-Value
Actin binding cytoskeletal protein	9.9	4.5E-10
cytoskeletal protein binding	11.3	2.3E-08
Cytoskeletal protein	12.6	7.5E-08
actin cytoskeleton	8.1	2.0E-07
actin binding	8.1	7.8E-07
ossification	4.1	1.1E-04
bone development	4.1	1.8E-04
contractile fiber part	4.1	1.8E-04
extracellular region	20.3	1.9E-04
BMP signaling pathway	2.7	1.9E-04
insulin-like growth factor binding	2.3	2.8E-04
contractile fiber	4.1	2.9E-04
Non-motor actin binding protein	4.1	4.3E-04
extracellular region part	12.2	5.0E-04
phospholipid metabolic process	4.5	6.3E-04
regulation of cellular component size	5.4	6.6E-04
regulation of cell growth	4.5	8.9E-04
organophosphate metabolic process	4.5	9.2E-04
Signaling by BMP	1.8	1.0E-03
adherens junction	4.1	1.5E-03
transmembrane receptor protein serine/threonine kinase signaling pathway	3.2	1.9E-03
basolateral plasma membrane	4.5	2.2E-03
Sphingolipid metabolism	2.3	2.4E-03
Extracellular matrix	5.4	2.9E-03
anchoring junction	4.1	2.9E-03
extracellular matrix	5.9	2.9E-03
cell adhesion	8.6	3.0E-03
Cell adhesion molecule	5.4	3.0E-03
biological adhesion	8.6	3.0E-03
cytoskeleton organization	6.3	3.4E-03

Supplementary Figure 1: Antiproliferative effect of MSC, expanded either on 3D-perfusion or 2D, on CD4+ activated cells.



Supplementary Figure 2: QRT-PCR evaluation of gene expression for selected genes to validate the microarray data. Legends: 3D represents the 3D-perfusion condition



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CHAPTER II

Thymus engineering: a 3D *in vitro* model to culture functional adult thymic epithelial cells

Elia Piccinini[1], Stefan Heiler[1], Caroline Berkemeier[2], David Wendt[1], Thomas Barthlott[2], Georg Hollander[2], Ivan Martin[1]

[1]Institute for Surgical Research & Hospital Management, University Hospital Basel, Basel, Switzerland

[2]Department of Biomedicine, University of Basel, Basel, Switzerland

Abstract

The lack of available models for *in vitro* culture of functional adult thymic epithelial cells (TEC) is a limiting factor for the engineering of a thymic organoid and the understanding of the development of a competent immune system. Therefore, we aimed to establish a 3D model for *exvivo* culture of functional TEC. We first demonstrated that a small number of primary adult murine TEC seeded onto a scaffold could support T-cell maturation *in vivo*, but to a limited extent. To increase the potency of the graft, we aimed to expand TEC *in vitro* . However, TEC expanded under conventional conditions (in 2D on plastic) lost their specific phenotype and functionality. In contrast, TEC expanded for 12 days in 3D in a fibrin hydrogel formed $EpCAM^+$ cell aggregates and showed phenotypic properties similar to freshly extracted TEC in terms of size and marker expression (e.g. MHC II). We have developed an *in vitro* 3D model that maintains the functionality of expanded TEC, paving the way to a better understanding of TEC/thymocyte cross talk and to unprecedented approaches for unraveling and controlling T cell development.

Introduction

Thymic epithelial cells (TEC) are the main component that constitutes the unique microenvironment that is required for the life-long formation of competent T cells [1]. Embedded in the three-dimensional architecture of the thymus, TEC and other stromal cells are responsible for attracting circulating hematopoietic cells and drive their maturation into functional T cells through highly regulated selection mechanisms, generally known as central tolerance induction [2,3]. The maturation process requires a journey through functionally distinct thymic areas, the cortex and the medulla, populated by TEC with different phenotypes (cTEC and mTEC, respectively). Hematopoietic progenitors colonize the thymus and, due to the inductive signaling provided by the stroma, undergo a series of differentiation steps characterized by phenotypically and functionally distinct intermediates. Concurrent expression of the T cell receptor (TCR) and the co-receptors CD4 and CD8 is the hallmark of the generation of thymic pre-T cells called double positive (DP) [4]. DP cells are then selected based on their ability of interacting with MHC I and MHC II – antigen complexes presented on the surface of TEC. The affinity of this interaction, unique for each cell due to the random TCR rearrangements, determines whether the developing T cell receive the necessary survival signals to complete maturation. As a result, CD4 or CD8 single positive cells (SP) exit the thymus and colonize the peripheral lymph nodes.

The newly generated T cells have a pivotal role in the establishment of a functional adaptive immune system by maintaining balance of immunity and tolerance [5]. Pathological deficits in thymus structure and function are associated with life-threatening phenotypes in both congenic (complete Di George Syndrome, Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy [APECED]) and acquired (Graft Vs Host Disease, chemo-radiotherapy, infections, malnutrition) clinical situations. These are responsible for significant defects of the adaptive immune system causing immunodeficiency, autoimmunity and an increased risk for

tumor relapse due to impaired immunological surveillance [6]. Moreover, thymic mass and function reach a plateau in puberty, than progressively decrease with age, leading to a diminished export of naïve T cells [7,8]. Known as thymic involution, this process of senescence is marked by a gradual displacement of epithelial tissue with adipose infiltrations, hence leading to a decrease in the frequency and absolute number of cTEC and mTEC. Nevertheless, the postnatal thymus displays a high turnover rate of TEC populations, suggesting the existence of a TEC population or progenitor that constantly replenishes cell loss [4–7], even if perinatal deletion of the thymus is not fully compensated later in life, suggesting limited regenerative capacity of thymic tissue [9–11].

However, despite its relevance, a limited understanding of TEC development and function hinders the investigation of the thymus. While in vivo studies seem to indicate the existence of a bipotent progenitor able to generate both cTEC and mTEC, the inability of prospectively isolate and culture functional TEC and the lack of information on their eventual niche, currently limits our comprehension of thymus biology [12–14]. In fact, TEC cultured ex vivo in conventional monolayer rapidly lose the expression of functional markers and thus their ability to support T cell development [15–17], with the notable exception of rat TEC that demonstrated high plasticity in a feeder layer system [18].

When grown in vitro as reaggregates, freshly isolated fetal TEC can create a microenvironment competent to support T cell development [12,19–21], thus providing a proof of principle that TEC grown under physiological conditions maintain their lymphopoietic capacity. All attempts to engineer a thymus organoid using postnatal TEC, their putative precursors or their expanded progeny have, however, been unsuccessful because the signaling and microenvironmental properties required to support postnatal TEC adherence and growth ex vivo while maintaining their functional capacity have yet to be identified. Hence, creating the conditions for postnatal TEC homeostasis and function constitute the first challenge towards the in vitro engineering of thymus and prospective regenerative strategies. This gap in the knowledge of thymic biology is cause and consequence of a lack of proper tools to culture TEC *in vitro*. Establishing an *in vitro* thymus model would provide a unique and indispensable tool for the efficient and detailed investigation of TEC and thymocyte biology at an organismic, cellular and molecular level. Knowledge

gained from such studies would provide essential insight into the mechanisms that control intrathymic T cell maturation and selection and are likely to accelerate the development of therapeutic strategies to regenerate thymus functions in vivo.

As known from the current tissue engineering approaches, the successful creation of tissue-like structures or organoids depends on the correct choice and design of bioengineered substrates. In particular, substrate mechanics and surface topography have been shown to dramatically influence cell proliferation [22] and dictate the lineage of cultured cells [23–25]. Hypothesizing that the development of cell-specific culture conditions is instrumental to manipulate TEC without losing their distinctive functionality, we hereby propose a tissue engineering approach to establish a model aimed to overcome the limitations of adult TEC cultures by providing 3D a scaffold based microenvironment that more physiologically mimics the cellular architecture of the thymus.

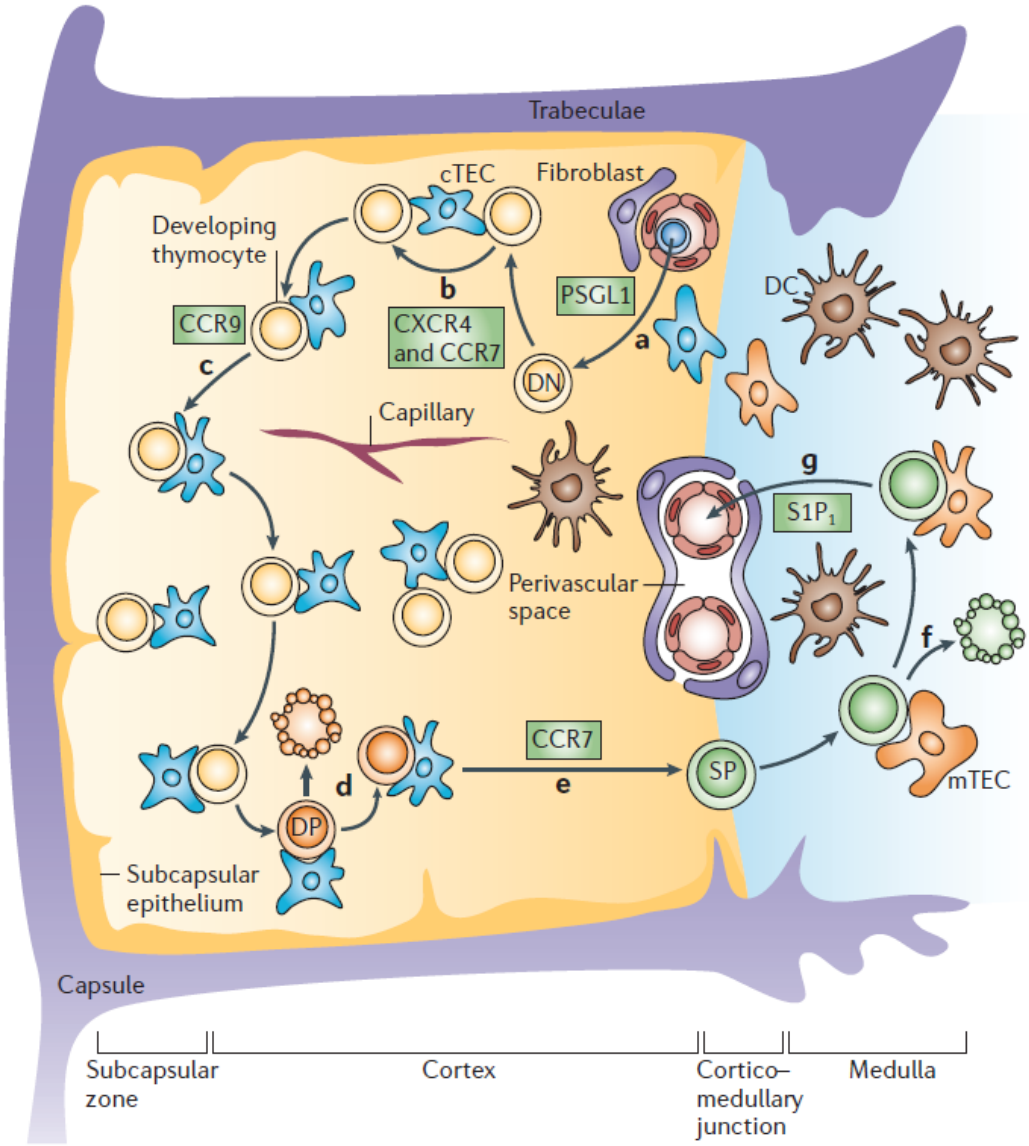

Figure 1

Model of T lymphoid development through the different compartments of the thymus. TEC display different phenotype and function depending on their location in the cortex (cTEC) or in the medulla (mTEC). Hematopoietic precursors enter the thymic microenvironment via blood vessels. Following their cTEC-instructed commitment to the T-cell lineage, these cells begin to mature through a step-wise process into distinct precursor populations, and eventually attain a phenotype marked by the concomitant expression of both CD4 and CD8 and the complete TCR/CD3 complex (double positive (DP)). The recognition of peptide-MHC complexes induces the differentiation into single positive (SP) CD4 or SP CD8 thymocytes according to their TCR recognition of MHC class II or class I molecules, respectively. DP thymocytes that fail to bind peptide-MHC complexes of either class will die by apoptosis, whereas sufficient recognition of MHC molecules will result in positive selection. Thymocytes migrate then to the medulla where they are exposed to negative selection by mTEC. SP thymocytes with a high affinity TCR for the peptide-MHC complex

will be deleted being a possible source of autoimmunity, whereas those with an intermediate affinity TCR will be exported to the periphery. (adapted from Takahama, Nature Reviews Immunology 2006).

Material and Methods

Mice

Thymic organs for preparation of thymic epithelial cells were isolated from adult B6.Ly5.1 mice between 4 and 7 weeks of age. Transplantation experiments with scaffolds grafted under the kidney capsule were done with B6.Foxn1nu/nu mice around the same age. All mice strains where obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and housed at the Center for Biomedicine's animal facility in accordance with institutional review board approval from the University of Basel and the Cantonal Veterinary Office.

Cell Isolation and Preparation

After Thymus dissection and removal of adherent connective tissue and fat, the lobes were digested at 37°C in a Phosphate-Buffered Saline (PBS) (pH 7.4; Invitrogen, CA, USA) solution containing Liberase (Roche, Basel, Switzerland) and DNAse (0.3 mg/ml; Roche, Basel, Switzerland) by passing the lobes through a 1000 µl pipet tip to promote dissociation. In the beginning the tip was cut for a larger opening. After allowing the organoids to settle, the supernatant was collected and new digest solution was added to the remaining organoids. This was repeated until a homogenous cell suspension was obtained that could be passed through a 10 µl pipet tip. Isolated cells were washed with FACS Buffer (PBS, 2 % FCS), spun down (1500 rpm, 5 min, 4°C), resuspended in FACS

buffer and pooled. Number of cells in suspension was determined using a Coulter Counter system (Z Series; Beckman Coulter, CA, USA) measuring particles with a size between 4.1 and 7.4 µm. For counting, a fraction (10µl) of the cell suspension was diluted in 10 ml Isoton II Diluent (Beckman Coulter, CA, USA) containing three drops of ZAP-Oglobin (Beckman Coulter, CA, USA).

Cell Separation

To enrich the fraction of $CD45^-$ stromal cells in the cell suspension, CD45 positive cells were depleted from the suspension using the autoMACS Pro Seperator system (Miltenyi Biotec, NRW, Germany). Cells were labeled by incubating 500 µl anti-CD45 microbeads for mouse (Miltenyi Biotec, NRW, Germany) per $8.0x10^8$ cells for 15 min at 4°C. After incubation, cells were washed in FACS Buffer, strained first with a 70 µm cell strainer (BD Bioscience, NJ, USA) followed by a second straining with a 40 µm mesh mounted on a 2 ml disposable syringe. Concentration of cells in suspension was adjusted to $1.0x10^8$ cells/ml and 200 µl of DNase (10 mg/ml; Roche, Basel, Switzerland) was added. For separation, the sensitive depletion program of the autoMACS was used. Finally, the non-labeled ($CD45^-$) cells in the negative fraction were spun down, resuspended in 1 ml of Iscove's Modified Dulbecco's Medium (IMDM) (Gibco; Invitrogen, NY, USA) containing 10 % FCS, β-mercaptoethanol and Kanamycin (Gibco; Invitrogen, NY, USA) and again counted as described above.

Culture Medium and Supplements

TEC were cultured in CnT-57 cell culture medium (CELLnTEC, Bern, Switzerland). CnT-57 is a progenitor cell targeted liquid culture medium with low calcium concentration (0.07 mM) and low concentrated Bovine Pituitary Extract (BPE) (6 µg/ml). CnT-57 is a

protein free medium containing a variety of amino acids, minerals, vitamins and other factors such as Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), and Insulin.

Additionally, the culture medium was supplemented with 10nM dihydrochloride monohydrate, a cell-permeable anti-apoptotic selective Rho-associated Kinase (ROCK) Inhibitor (10nM; Calbiochem, Merck, Darmstadt, Germany) to enhance viability. Kanamycin was supplemented to the medium to reduced potential hazard of contamination.

Fibrin gel

Fibrinogen Type I-S was obtained from Sigma-Aldrich (MO, USA), which consists of 65 - 85% protein, of which more than 75% is clottable. The lyophilized powder was reconstituted with 0.9% saline solution (NaCl) to a concentration of 100mg/ml. For a working solution with a concentration of 40mg/ml, the fibrinogen solution was further diluted either with 0.9% NaCl or aprotinin (3000 KIE/ml; Baxter, IL, USA). For the generation of the fibrin gel, equal volumes of fibrinogen and thrombin (approximately 50µl each) were mixed together to induce crosslinking.

2D Cell Culture

TEC were expanded by conventional 2D monolayer culture (i.e., on tissue culture plastic) at a density of $4x10^4$ TECs/cm2 of growth area resulting in $3x10^5$ TECs for a 48-well-plate. Cells were cultured in 200µl medium, with medium changes every second day. For cell harvest, 0.25% Trypsin was used to detach the cells from the plastic.

3D Cell Culture Models

3D scaffold-based dynamic culture model (fetal TEC). As proof of principle, we first established a 3D model system based on fetal TEC and a dynamic bioreactor culture system. Murine thymi were harvested from 13 day old embryos and digested to obtain a cell suspension. TEC were separated by flow cytometry as EpCAM-1^{+} (Epithelial cell adhesion molecule) CD45^{-}cells, then loaded in 2 perfusion bioreactors (2x10^{4}cells/bioreactor) to seed and culture the cells on UltrafoamTM (Davol Inc.), a 3D porous collagen scaffold (Ø=3mm). TEC-scaffold constructs were cultured under perfusion for one week in IMDM supplemented with Epithelial Growth Factor.

Freshly seeded 3D scaffold model (adult TEC): We next assessed whether adult TEC, which have been extracted from the thymus (i.e., removed from their native microenvironment and its associated cues), could maintain their functional capacity if reintroduced back into a 3D environment. Adult TEC were isolated and separated as described above. Immediately following separation, TEC were loaded into Ultrafoam scaffolds. Without an *in vitro* culture phase, TEC-scaffold constructs were immediately implanted into the kidney capsules of athymic mice for 6 weeks.

3D fibrin model (adult TEC): For the 3D cell culture model, 2x10^{5} TECs were embedded into fibrin gel with a total volume of 100µl. Fibrinogen and thrombin were diluted as previously described to a concentration of approximately 40 mg/ml and 10 U/ml, respectively, and mixed in equal volumes in a pipet tip. TEC, resuspended in 10µl IMDM, were gently mixed with the fibrin solution by pipetting. The cell/fibrin mixture was slowly (to avoid bubble formation) pipetted onto a sterile parafilm disc (12mm diameter), which was placed at the bottom of the well (24-well-plate). After 15 minutes incubation at 37°C, when the polymerization was complete, 1 ml of culture medium was added to cover the cell/fibrin construct. Medium was changed every second day.

To harvest the cells, fibrin constructs were treated with 0.25% Trypsin EDTA until all fibrin was digested.

Cell Proliferation Assay

To measure the proliferation of TEC in monolayer 2D culture, cells were plated at a density of $4x10^4$ cells/cm^2 in a 96-well-plate ($1.28x10^4$ cells/well). TEC proliferation was assessed for three weeks at two day intervals. For the first two time points, the supernatant was collected and treated separately to quantify unattached cells. For reference, corresponding cell numbers were collected on day 0 (day of seeding). All time points were assessed in triplicates. For each time point, the supernatant was removed from the culture well (except for the first two time points) and the adherent cells were incubated in 100µl proteinase K solutions (Proteinase K 1mg/ml, pepstatin A 10µg/ml, EDTA 1mM, iodoacetamide 1mM, 50mM Tris pH7.6; Sigma-Aldrich, MO, USA) over-night. The next day, cells were collected in separate wells and stored frozen at -20°C until the collection of all time points was complete. DNA quantification was performed with the CyQUANT™ Cell Proliferation Assay (Gibco, Invitrogen, NY, USA) according to the manufacturer's protocols.

TEC Phenotype

TEC gene expression was assessed at different stages of the TEC isolation process and after one week of *in vitro* culture. The stages of the isolation process include the whole organ, the digested organ, the CD45 depleted cell suspension, cells sorted for $EpCAM^+$ and cells cultured *in vitro* for one week sorted for MHC^+ and MHC^-, respectively. Total RNA extraction was performed with the RNeasy Micro Kit (QIAGEN, Basel, Switzerland) according to manufactures protocol. Reverse transcription into cDNA was

done with Oligo (dT20) or random-N6 primers and reverse Transcriptase 3 (Invitrogen, CA, USA) following the standard protocol with the Mastercycler Gradient (Eppendorf, Hamburg, Germany). For quantitative real-time PCR, the Sybr-Green method was used (SensiMix; Quantace Biolabo, Châtel St. Denis, Switzerland) for the following oligonucleotides sequences:

EpCAM: fw: TGAGGACCTACTGGATCATC; rv: TATCGAGATGTGAACGCCTC

FoxN1: fw: GTGGAACTGGAGTCCACG; rv: TGTTGGGCATAGCTCAAGCC

β5t: fw AACAAACAAACTCCCAAACC ; rv: CTCTCCTCATTTGCTTCTTCAG

Aire: fw: CCAGTGAGCCCCAGGTTAAC; rv: GACAGCCGTCACAACAGATGA

Ccl19: fw: CCTGGGTGGATCGCATCATCCG; rv: AGAGCATCAGGAGGCCTGGTCCT

Ccl21: fw: AGCTATGTG-CAAACCCTGAGGA; rv: GAAAGCCTTCCGCTACCTTCTT

Ccl25: fw: GTTACCAGCACAGGATCAAAT; rv: GGAAGTAGAATCTCACAGCA

Dll4: fw: GGAAATGGCATGAACTGAAC; rv: GATGCTCCTCTAACTCCTCAAC

K5: fw: TCAACAAGCGTACCACGGC; rv: GGCATCGACCCTGGCC

K8: fw: GCCACTGAAGTCCTTGCCAG; rv: GGTTGGCCAGAGGATTAGGG

GAPDH: fw: ACCATGTAGTTGAGGTCAATGAAGG; rv: GGTGAAGGTCGGTGTGAACG

Specificity of the PCR was controlled by melting curves measurements and agarose gel electrophoresis of the PCR product. The ΔΔCt method was used for the quantification of the PCR reaction and amounts of specific mRNA was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or EpCAM (Epithelial cell adhesion molecule).

TEC Functionality

In vitro positive selection of thymocytes: To assess the maintenance of the TEC's capacity to induce T-cell differentiation, we used an *in vitro* co-culture model to assess

thymocyte positive selection. In brief, $1x10^5$ cultured TECs and $1x10^5$ $CD4^+/CD8^+$ (DP) Thymocytes (sorted for $CD69^-$) were pooled together in a 0.5 ml reaction tube (Eppendorf, Hamburg, Germany) and spun down (4500rpm, 1 min, RT) in a tabletop centrifuge (5415R; Eppendorf, Hamburg, Germany). Reaggregated pellets were cultured in the tube in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Invitrogen, NY, USA) supplemented with 10% FCS and 10 nM ROCK Inhibitor for 48h in an incubator. After 48h, aggregates were dissociated and thymocytes were analyzed by flow cytometry for the expression of CD69, CD4, CD8 and T-cell receptor (TCR).

In vivo functionality of TEC: Maintenance of TEC functionality was assessed *in vivo* by ectopically implanting scaffolds, fibrin constructs, or reaggregated TEC under the kidney capsule of athymic mice. After 6 weeks, implants and lymph nodes were harvested and digested with liberase to extract thymocytes. T-cell development (CD4 and CD8 double positive or single positive cells) in the implants and in the peripheral nodes was assessed by means of flow cytometry.

Flow Cytometry

Flow cytometry was performed on a FACS ARIA II Special Order System (BD Bioscience, CA, USA) and acquired data were analyzed using FlowJo software (TreeStar, OR, USA). Sample preparation for flow cytometry was done according to standard protocol. In brief, cells in suspension were incubated with antibodies on ice for 45 minutes in the dark, spun down, washed and resuspended in FACS Buffer (PBS, 2% FCS) and acquired. Cells were stained using directly labeled antibodies against CD45, EpCAM (G 8.8), Ly51, UEA-1 and major histocompatibility complex (MHC) II (I-Ab) for the phenotypical characterization after isolation and after culture. For the determination of cell viability,

DAPI was added prior to acquisition. Antibodies were obtained from either from BD Bioscience or eBioscience (San Diego, CA, USA)

Transduction of TECs in a 3D Environment

To demonstrate the feasibility of genetically modifying TEC directly within our 3D model system, and for tracing of the cells during time lapse microscopy, TECs were transduced with a lenti viral vector for expression of Green Fluorescent Protein (GFP). In brief, cells were embedded into fibrin gel and cultured as described above. Five days after embedding, cells were transduced with a multiplicity of infection (MOI) of 30 transducing units per cell. Cells were incubated over-night in the virus containing medium (CnT-57, 10 µM RockI, 8µg/ml Polybrene). First signs of GFP expression were observed after 24h with a fluorescent microscope and time lapse experiments were started five days post transduction

Results

Evaluation Of A 3d Scaffold Based Approach For Fetal Tec Culture

According to previous reports, fetal TEC from D14-D16 mice embryo can be consistently cultured in micromasses at the air/liquid interface, maintaining their thymopoietic capacity *in vitro* [19]. Due to this feature, fetal TEC were used in combination with a collagen-based biomaterial as a cell source to validate the scaffold-based approach (Fig. 2).

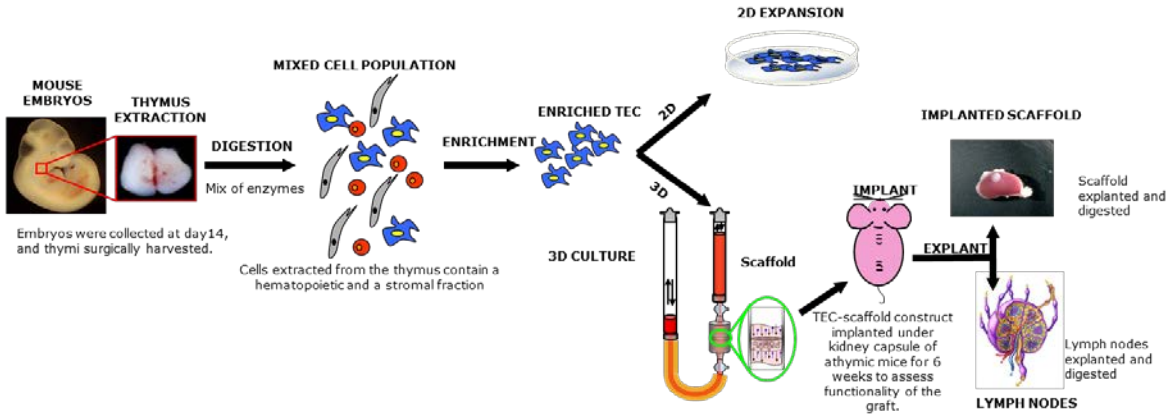

Figure 2

Experimental setup to harvest and culture TEC. Thymi are harvested from embryonic or adult mice and enzymatically digested to obtain a single cell suspension. Cell suspension can then be enriched in TEC frequency by FACS or magnetic beads. TEC are now ready to be cultured, either on a 3D scaffold (in this case the supplemental use of a perfusion bioreactor is depicted) or on a traditional plastic substrate. The 3D construct is then implanted under the kidney capsule of an athymic mouse. After 6 weeks, construct and lymph nodes are extracted and presence of respectively DP and SP investigated.

In order to achieve homogeneous cell density and avoid the formation of undesirable gradients of nutrients and waste-products, cells were seeded and cultured in an oscillating perfusion bioreactor [26]. The scaffold consisted in a porous collagen sponge (Ultrafoam ®). After 1 week of culture, the construct was implanted under the kidney capsule of athymic mice to assess cell functionality. Athymic mice with Foxn1 mutation are not competent to develop mature thymocytes, although few T-cells of extra-thymic origin can be detected in the periphery. However, these mice typically display a non-physiological ratio of $CD4^+$ on $CD8^+$ SP cells of extra-thymic origin. In fact, while in wild type mice $CD4^+$ cells are approximately the double compared to $CD8^+$ cells, in athymic mice this ratio decreases due to a higher proportion of $CD8^+$ cells. Restoration of thymic-derived lymphopoietic activity due to the presence of a functional thymic graft increases again the $CD4^+/CD8^+$ ratio above 2. Negative control

consisted in mice transplanted with a TEC-free scaffold, while a wild type (non-nude) mouse was used as a positive control.

Six weeks upon implantation, graft and lymph nodes were harvested from the transplanted recipients, and the lymphoid population analyzed. Substantially no DP thymocytes were recovered from the negative control, and peripheral lymph nodes consistently showed few T-cells of extra-thymic origin (Fig 3a). Remarkably, mice that received the TEC^{-}based construct exhibited 46.4 % of DP thymocytes recovered from the graft (Fig. 3b). The capacity of the graft to support lymphopoiesis was further demonstrated by the detection of and $CD4^{+}$ and $CD8^{+}$ SP in a ratio >2 in the peripheral lymph nodes, underlying the thymic origin of these mature T-cells (Fig. 3b). Cells harvested from the lymph nodes were pre-gated on TCR expression as a marker for mature T-cells. Additionally, memory T-cells were also detected in the periphery. However, comparison with wild type positive control shows that (Fig. 3b, 3c) thymocytes originated in the engineered organoid were less abundant (Fig. 3b, 3c).

This approach demonstrated that 3D scaffold-based cultures could represent a viable alternative for aggregate cultures, although adding a remarkable degree of plasticity with regard to the tunable properties and allowing immediate scalability.

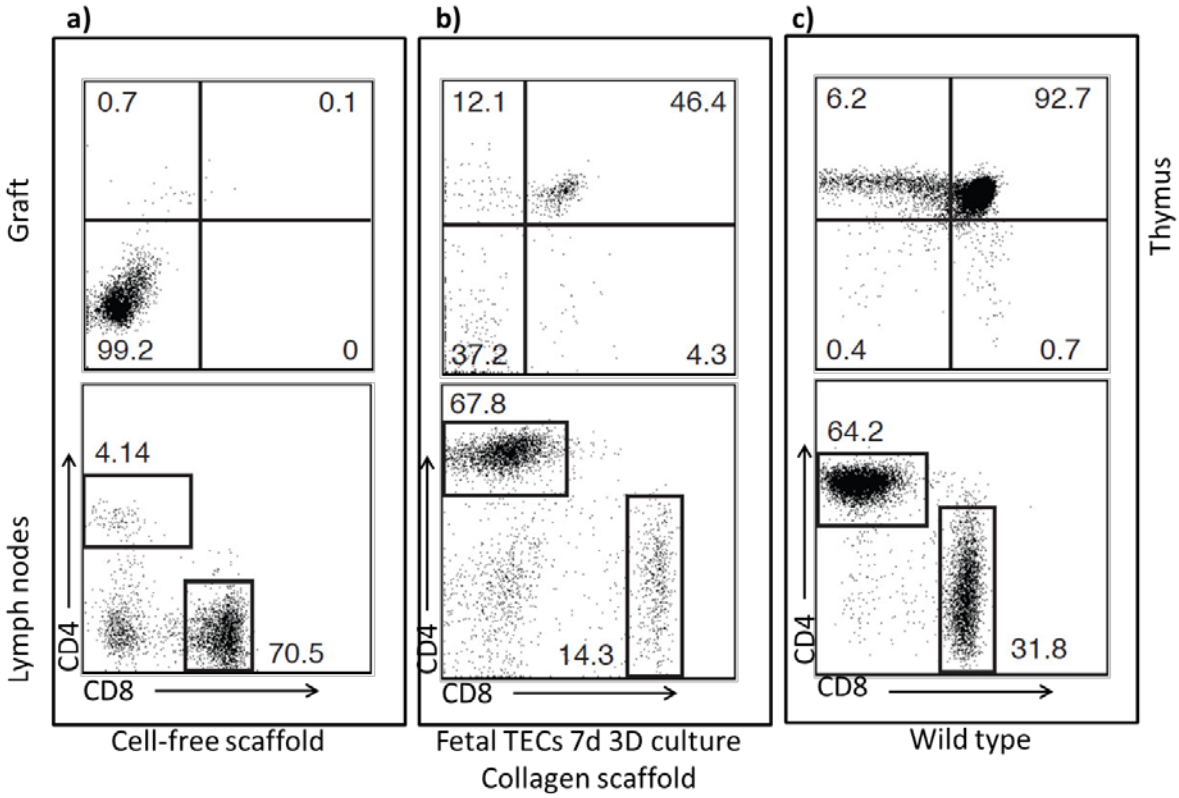

Figure 3

Analysis of cells recovered from explanted grafts and lymph nodes after 6 weeks implantation into athymic mice. Cell-free scaffold used as a control was not able to promote lymphopoiesis (2a). Graft with cultured TEC contains a consistent number of developing DP thymocytes, and peripheral nodes are repopulated with TCR$^+$ SP with a ratio CD4$^+$/CD8$^+$ >2 (2b). Thymus and lymph nodes from wild type mouse as a positive control (2c).

Manipulation Of Adult Tec

Despite representing a valuable model for TEC cultures we reasoned that fetal TEC do not represent a relevant cell source as compared to post-natal TEC, in consideration of our attempt of ultimately recapitulating a model for the investigation of post-developmental biological processes or for prospective therapies targeting adult thymus regeneration.

As a consequence, we decided to translate the successful 3D culture approach to the use of adult TEC that had so far failed to be cultured *in vitro* maintaining their

functionality. In fact, adult TEC are intrinsically unable of forming aggregate cultures of pure TEC (thus lacking the possibility of self-reconstructing a 3D stroma as fetal TEC do), and tend to lose their differentiation status and capacity of supporting lymphopoiesis when cultured *in vitro* on plastic.

In order to assess the feasibility of a scaffold-based approach with adult TEC, thymi of 5 week-old mice were dissected and the $EpCAM^+$ cells sorted to be immediately seeded on a collagen scaffold (used as a carrier) and implanted under the kidney capsule of athymic mice, thus avoiding any culture step. After 6 weeks, scaffolds and lymph nodes were explanted for analysis. Similarly to fetal, adult TEC were able to sustain the maturation of lymphoid progenitors in DP cells in the implanted graft (Fig. 4a). Moreover, differentiated SP cells were ultimately able to exit the graft and home to the peripheral lymph nodes, as demonstrated by the increased $CD4^+/CD8^+$ ratio (Fig. 4a).

Despite consistent, the overall process of maturation of lymphoid precursors to thymocytes displayed a lower efficiency in terms of numbers when compared to the wild type or even if compared to fetal cells (Fig. 4b). It was therefore hypothesized that the potency of the implanted graft needed to be increased to recapitulate the cell density of the native thymus and thus achieve a more extensive generation of mature thymocytes. In particular, we aimed at boosting the lymphopoietic capacity of the implanted graft by increasing its cellularity.

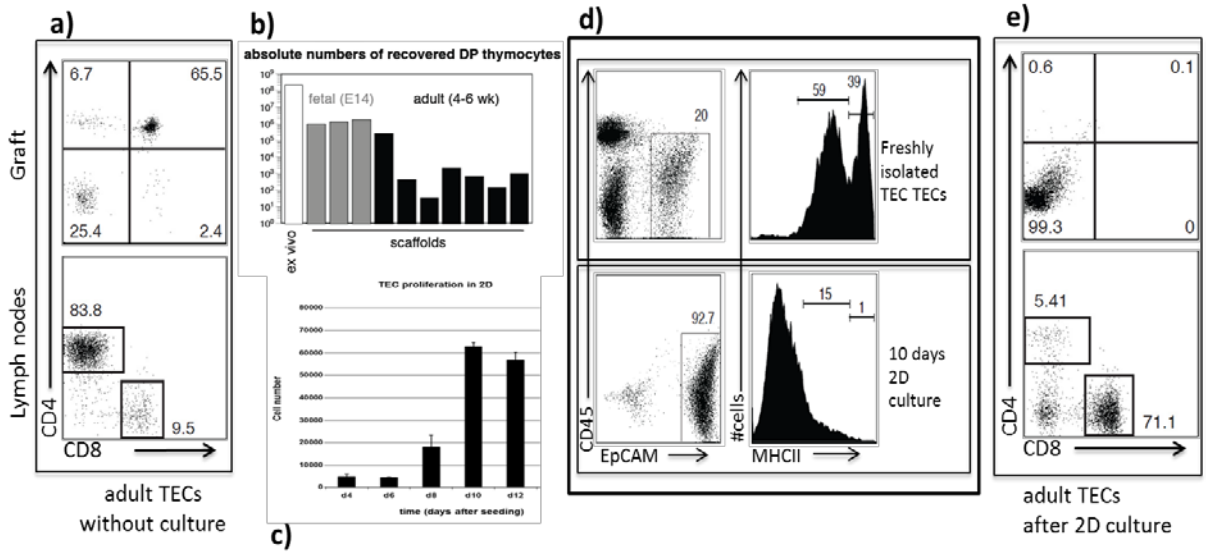

Figure 4

Analysis of TEC functionality and phenotype at different manipulation stages. Postnatal TEC can be explanted and immediately re-implanted maintaining their T-cell maturation functionality (3a). Their lymphopoietic capacity is reduced if compared to fetal cells (3b). Selected culture conditions in 2D allow cell proliferation in culture (3c), and the selection of a highly enriched $EpCAM^{+}/CD45^{-}$ population that display a marked decrease of MHC II expression (3d). Cells cultured in 2D fail to support lymphopoiesis when re-implanted *in vivo*.

According to the tissue engineering paradigm based on cell manipulation *in vitro*, we wanted to achieve the increased cellularity by expanding the cells *ex vivo* rather than increasing the number of the donor mice.

Our first step consisted in testing different media for cell culture and their supplementation with specific growth factors. In consideration of the need of easily evaluating the progression of the culture and testing many conditions in parallel, screening of the culture media was carried out in 2D.

Finally, a specialized medium for epithelial cells was selected because of its proliferative effect and selectivity in preventing the expansion of $EpCAM^{-}$ cell populations that typically overgrows TEC in other serum-containing culture media.

Growth kinetic of TEC displayed a lag-phase until days 6-8 when proliferation starts, until reaching confluence in the plate with a 12-fold increase of cell number (Fig. 4c) and a $EpCAM^{+}/CD45^{-}$ purity above 90%. However, expanded $EpCAM^{+}/CD45^{-}$ cells displayed a significant change in MHC II expression, from 59% MHC II $^{+/low}$ and 39% MHC II $^{+/high}$ of the freshly isolated cells to 15% MHC II $^{+/low}$ and 1% MHC II $^{+/high}$ (Fig. 4d).

Expression levels of some key molecules like K5, K8, AIRE, Foxn1, and β5t in 2D cultured $EpCAM^{+}$ cells compared to freshly isolated TEC^{+} confirmed a general de-differentiation pattern intended as loss of tissue specific genotypical and phenotypical traits (Fig. 5). Moreover, it also evidenced how the process of harvesting cells from the thymi resulted in a remarkable loss of key transcripts, possibly due to the high mortality of TEC associated with the harvesting preocedure.

The phenotypical change reflected an actual loss of function as assessed by the inability of expanded TEC to restore a thymic function when seeded on a collagen scaffold and implanted under the kidney capsule. Interestingly, and again differently from fetal TEC, adult expanded $EpCAM^{+}$ cells failed to form a pellet stable enough to be handled when spun down in an eppendorf tube and incubated overnight.

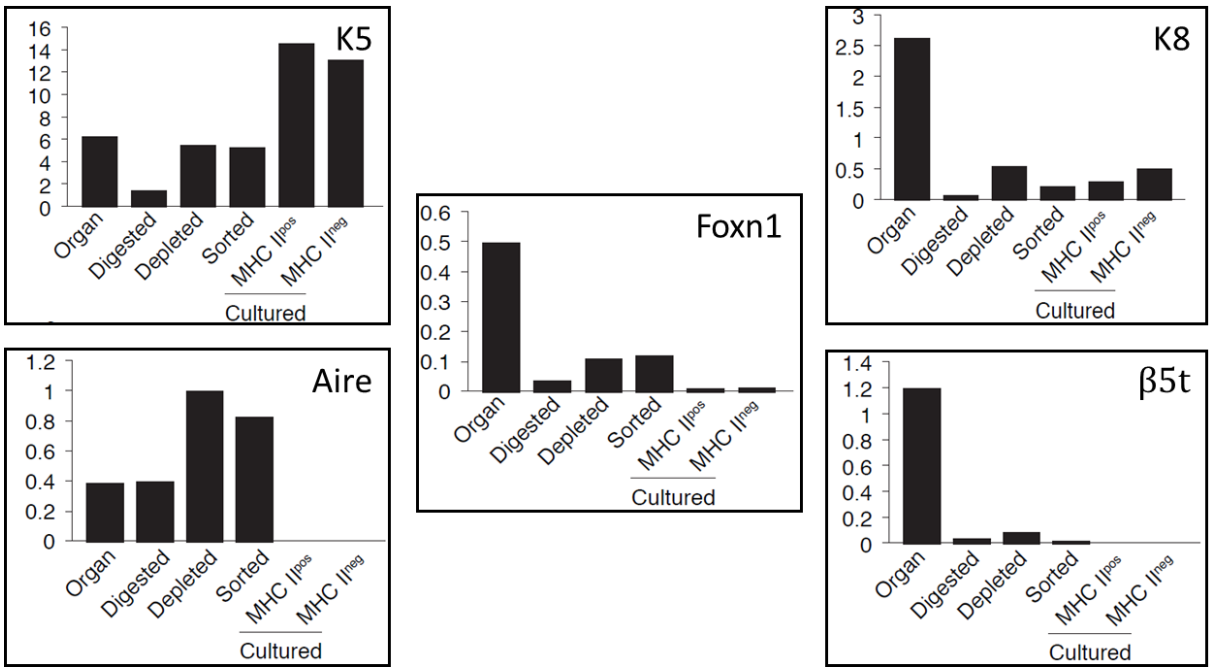

Figure 5

Expression levels of specific TEC genes. With the exception of K5, specific for mTEC, TEC culture leads to a change in the transcriptional stage of specific markers. It is also evidenced how extraction of TEC from the thymus (digested and depleted histograms) severely affects some TEC subpopulation.

Development Of A 3d Scaffold Based Culture System To Enable Manipulation Of Adult Tec

Next, it was hypothesized that 3D spatial organization of cultured cells and a scaffold with decreased stiffness might positively affect the maintenance of the phenotype of cultured TEC [27–30]. Fibrin glue hydrogel was thus chosen because of its reported extensive use with many cell types, cytocompatibility, and prospectively for its tunable chemo-physical properties [27,31,32].

Beyond the previously described 2D culture on plastic substrates, fibrin was then used in three different configurations: (i) as a thin-layer coating on the plastic surface to assess the influence of the binding of fibrin to adhesion molecules on the cell surface but maintaining the same substrate stiffness, (ii) as a thick substrate to investigate the

effect of a reduced stiffness substrate in addition to binding of adhesion molecules, and (iii) as an embedding medium to test the importance of a 3D architecture (Fig.6a). Cultured cells were analyzed at FACS. Interestingly, after 11 days of culture, cells displayed a different size pattern as measured by forward scatter at the FACS (Fig. 6b). Cells cultured on plastic and fibrin-coated plastic tended to increase their size compared to freshly isolated TEC. However, when cells were grown on the soft fibrin-bed the increase was smaller. Finally, 3D embedded cells showed the best resemblance to the original size (fig.6b). Moreover, expression of MHC II on cells cultured on plastic and coated plastic was reduced to less than 5% of positive cells. Cells growing on the thick substrate scored a 7.5% of MHC II^{+}, while embedded cells displayed a population of 57% MHC $II^{+/low}$ cells. Despite the difference in size and the loss of the fraction of $EpCAM^{+}/CD45^{-}$/MHC II^{high} in all samples when compared to freshly isolated TEC, embedded cells showed an intermediate phenotype between plastic cultured cells and freshly isolated cells that intriguingly suggest a better maintenance of TEC native properties. Because we wanted to demonstrate that cells can sense and respond to the 3D architecture of the environment, TEC were stained with a fluorescent dye and visualized at the microscope. In fact, cultured cells displayed a variety of morphologies, possibly underlying the preservation of different subpopulation, and created a complex network through elongated filaments providing contact with neighboring cells (Fig. 6c). With the progression of time, cells aggregated into dense clumps.

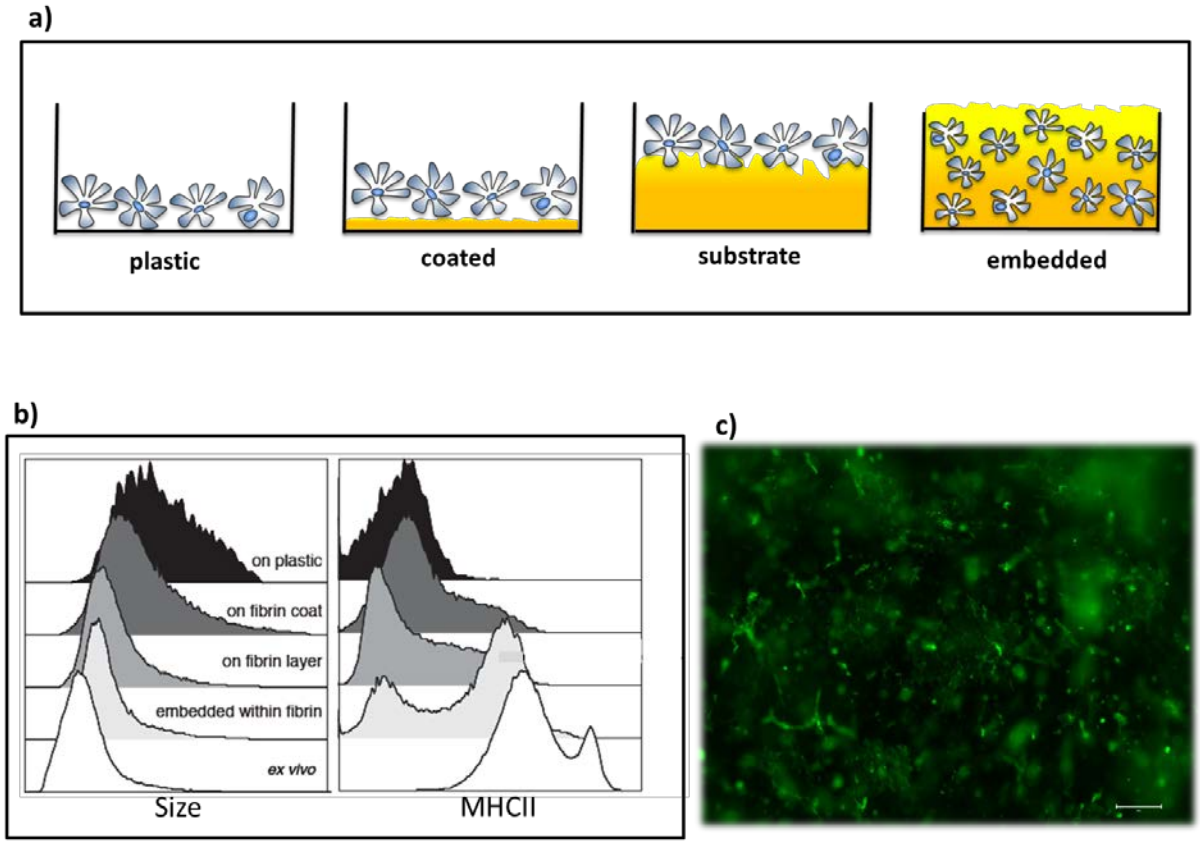

Figure 6

Different substrates and geometries influence the phenotype. Freshly isolated TEC were seeded on plastic, on plastic coated with a thin layer of fibrin, on a thick fibrin substrate, and encapsulated into the fibrin gel (5a). Analysis at FACS of Forward Scatter reveals a shift towards an increased size of cultured cells, however less marked for embedded cells (5b). MHC II expression among different culture conditions; although MHC II^{high} population is strongly reduced, embedded cells maintain a remarkable percentage of MHC II^{low} population (5b). Embedded TEC stained with a fluorescent marker present different morphologies enabling the establishment of a 3D network and a complex cellular architecture (5c).

In order to validate a functional assay that did not require the long time needed for the *in vivo* functional tests, a positive-selection assay for cultured cells was proposed as a surrogate for quick evaluation of cell functionality. The advantages of this *in vitro* assay also include reduced animal use and more control due to defined cellular input. As assessed with freshly isolated cells co-cultured in a pellet for two days with sorted $CD69^{-}$ DP thymocytes, only functional TEC are able to activate the thymocytes.

Activation is evidenced by an increased expression of CD69, and confirmed by a slight average decrease in the positivity of CD4 and CD8 expression. This sequence of events follows the interaction of the developing thymocyte with MHC I or MHC II molecules presented on competent TEC surface. Thymocytes that do not interact with competent TEC undergo apoptosis.

After culture in the previously mentioned culture conditions, TEC were and prepared for the assay. 10^5 TEC and 10^5 sorted DP/CD69$^-$ cells was pelleted in Eppendorf tubes to enable cell to cell contact. After 2 days, cells were stained and analyzed. A positive control carried out with fetal TEC demonstrated activation on approx. 14% of the total input (data not shown). As expected, cells cultured on plastic did not form any aggregate (Fig. 7a). Cells cultured on the thin layer of fibrin were able to activate by selection only 0.1% of the initial cells, and the overall viability of the thymocytes was severely impaired as shown by a decreased mean fluorescence of CD8 and CD4 of CD69$^-$ cells. Phenotype of CD69$^+$ thymocytes was not measurable (Fig. 7a). Interestingly, a 10-fold increase (1.1%) of the percentage of activated thymocytes was supported by TEC cultured on the thick fibrin layer. Among this subset of CD69$^+$ cells, 24% decreased the expression of CD4 and CD8, underlying that the physiological positive selection was occurring (Fig. 7a). Finally, TEC embedded in fibrin supported the activation of 4.2% of thymocytes; of this population, 34% decreased the expression of CD4 and CD8 (Fig. 7a). As a further confirmation of a possible increased functionality of fibrin embedded TEC, we calculated the total amount of viable thymocytes recovered from the Eppendorf tubes (Fig. 7b). Embedded TEC demonstrated that a higher number of viable thymocytes were maintained in culture, indicating an overall enhanced maintainance of stroma-like and thymopoietic functionality after culture compared to 2D cells.

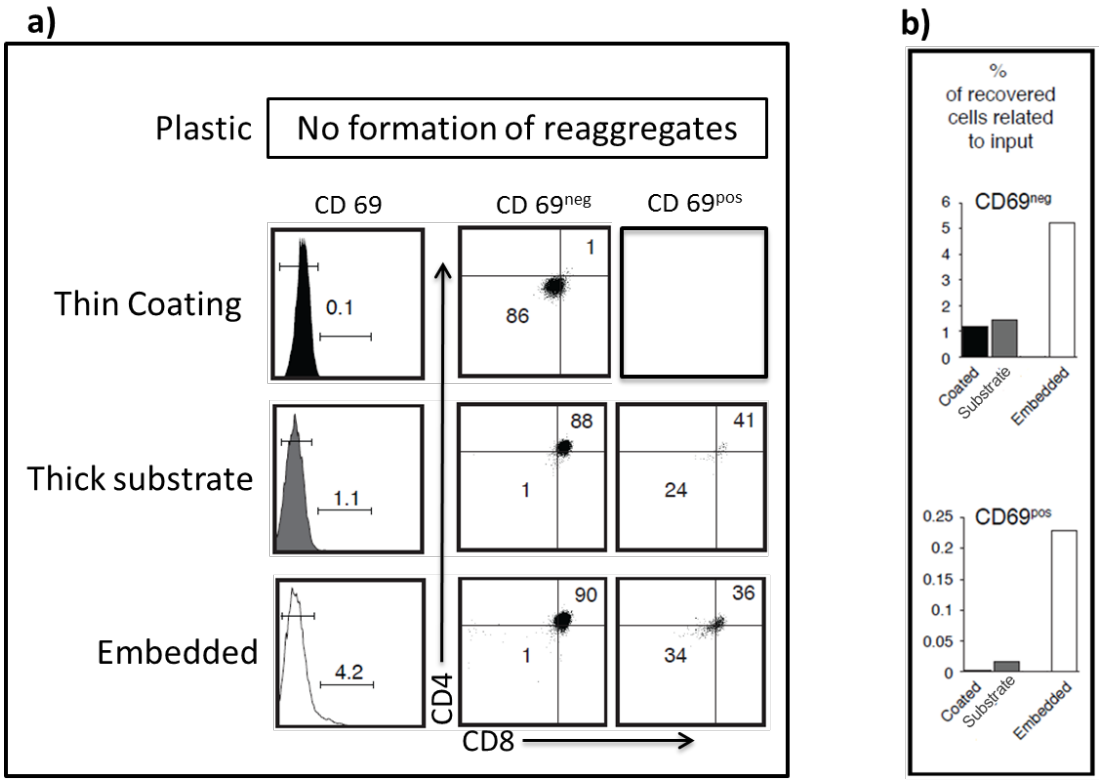

Figure 7

In vitro functional test based on positive selection of DP CD69^{-} cells. TEC cultured on different substrates display different supportive capacity to activate and maintain thymocytes. TEC grown on plastic do not reaggregate (6a), while TEC grown in presesence of fibrin can aggregate. Plastic and thin-coating cultured cells fail in activating and maintaining viable the DP thymocytes, while thick substrate and embedded cells can still support thymocytes activation (6b).

Finally, we wanted to challenge cultured TEC as a potential target for a gene therapy. In fact, the possibility of genetically manipulating a functional *in vitro* thymic organoid would enable numerous experiments bypassing the need of creating suitable animal models. Thus, we transduced with a lentiviral vector carrying GFP fibrin embedded cells. Live cells became visible at the confocal microscope showing high-motile cells, the formation of a complex 3D architecture and positivity for EpCAM upon immunostaining (fig. 8).

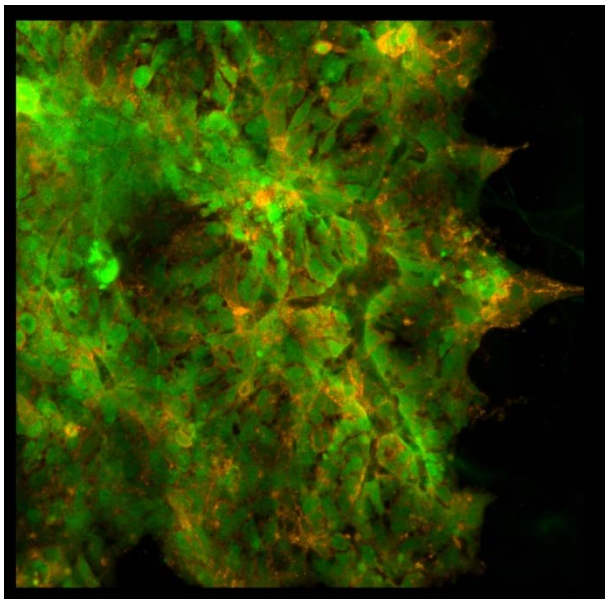

Figure 8

Confocal image of GFP transduced TEC embedded in fibrin. Fluorescence allows to visualize cultured cells while they assume a complex 3D architecture. Green= GFP. Orange= EpCAM.

Conclusions And Discussion

The approach we describe brings together multidisciplinary expertise under the common aim of developing a culture model for the thymus, a crucial organ for the definition of immunity and tolerance. First, the demonstrated that TEC, as the main player of the lymphopoietic functionality of the thymic stroma, can be cultured *in vitro* on a scaffold by using a robust cell model previously described, i.e. fetal TEC. Fetal TEC were cultured one week, and demonstrated to support thymocytes maturation once implanted in athymic mice. Then, arguing that postnatal TEC would represent a more relevant cell source for the understanding of thymic physiology, we tested the feasibility of maintaining adult TEC function by explanting and re-implanting

ectopically sorted TEC using a scaffold as a carrier. The consistency and at the same time the limited extent of the thymopoiesis was interpreted as a need to engineer the implantable graft increasing the number of delivered functional TEC. The achievement of a good proliferation of TEC in 2D was made inoperable by the loss of functionality, triggering the development of a fibrin based 3D hydrogel to use as a scaffold. Indeed, embedding the cells in fibrin resulted in the manufacturing of a cell-based construct where cells maintained a phenotype that resembled more the appearance of freshly isolated cells, and a limited but consistent functionality as assessed by *in vitro* assays. Finally, cells were successfully transduced with a lentiviral vector, enabling easy imaging of live cells while organizing in a complex 3D network.

Undoubtedly the proposed 3D model requires further *in vivo* validation, currently ongoing and providing promising results but needing confirmation on additional experiments. Additionally, cultured TEC are still not able to fully recapitulate their native lymphopoietic capacity, advocating the urge of a further engineering of the graft to be able to better recapitulate the thymic stroma.

Thus, we propose that two different approaches should be pursued to unveil the complex physiology of the thymus: (i) better investigating the influence of the single chemo-physical parameters on the phenotype and the functionality of cultured TEC, and (ii) increasing the complexity of the artificial stroma by designing specific 3D substrates and eventually complement the culture system with the other cellular (e.g. fibroblasts, macrophages, dendritic cells, lymphoid tissue inducers, and the thymocyte themselves) and acellular (soluble factors, extracellular matrix, organomorphic architecture) components that constitute, define, and maintain the thymic microenvironment [33].

Altogether, the reported data underline a promising advancement in the manipulation of functional postnatal TEC, ultimately proposing an innovative approach in investigating thymus physiology and paving the way to the engineering of the thymus for research and therapeutic applications.

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CHAPTER III

Toward modeling the bone marrow niche using scaffold-based 3D culture systems

Abstract

In the bone marrow, specialized microenvironments, called niches, regulate hematopoietic stem cell (HSC) maintenance and function through a complex crosstalk between different cell types. Although in vivo studies have been instrumental to elucidate some of the mechanisms by which niches exert their function, the establishment of an in vitro model that recapitulates the fundamental interactions of the niche components in a controlled setting would be of great benefit. We have previously shown that freshly harvested bone marrow- or adipose tissue-derived cells can be cultured under perfusion within porous scaffolds, allowing the formation of an organized 3D stromal tissue, composed by mesenchymal and endothelial progenitors and able to support hematopoiesis. Here we describe 3D scaffold-based perfusion systems as potential models to reconstruct ex vivo the bone marrow stem cell niche. We discuss how several culture parameters, including scaffold properties, cellular makeup and molecular signals, can be varied and controlled to investigate the role of specific cues in affecting HSC fate. We then provide a perspective of how the system could be exploited to improve stem cell-based therapies and how the model can be extended toward the engineering of other specialized stromal niches.

Introduction

Stem cells are self-renewing, multipotent progenitors which are responsible for the maintenance and turnover of several tissues. In the past few years, efforts have been directed at localizing stem cells in vivo and at identifying the specialized microenvironment, the “stem cell niche”, which regulates stem cell quiescence, self-renewal and differentiation. The term “niche” was used for the first time by Schofield in 1978 to describe the bone marrow (BM) environment in which hematopoietic stem cells (HSC) reside [1]. Since then, stem cell niches have been experimentally identified also for other tissues, like the nervous system [2], the skin [3] and the intestine [4], validating the general concept that environmental factors, including soluble signals, specific cellecell and cell-matrix interactions, as well as structural cues, are crucial for stem cell maintenance and function.

So far, HSC remain the best-characterized adult stem cells and several studies have aimed at identifying the main bone marrow niche regulators and unravelling the

molecular pathways that mediate the HSC niche function. Osteoblasts were shown to be of great importance for controlling the HSC niche size [5e7], but other cell types, like osteoclasts, endothelial cells and mesenchymal progenitors (reviewed in [8]) have also been involved in modu- lating HSC fate. In addition to the cellular elements, several mole- cules, including membrane bound stem cell factor (SCF) [9,10], osteopontin [11,12], trombopoietin (TPO) [13], stromal derived factor-1 (SDF-1) [14] and N-cadherin [5] as well as other factors, like low Ca2þ and oxygen concentrations [15e17], have been shown to affect HSC behavior and to support the establishment of the niche environment. Experimental evidences suggest that specific signaling pathways, such as Notch and Wnt, are activated in the bone marrow and they may have a role in HSC regulation [18]. Despite the success in identifying some of the components and signals of the HSC niche, the complete understanding of the mechanisms involved in HSC regulation still remains elusive, also due to the limited availability of appropriate ex-vivo models which could mimic the complex niche organization. Cell culture systems developed so far do not entirely reproduce the physiological signals required to establish a functional niche structure, resulting in a limited efficiency in maintaining long-term repopulating HSC in vitro.

In this paper we will describe scaffold-based 3D culture systems as potential tools to mimic ex-vivo the main components of the bone marrow environment. We will underline the potential advantage, as compared to traditional monolayer or suspension cultures, in providing a more physiological 3D structure which may be tailored thanks to the biomaterial design. Furthermore, we will highlight the possibility to control and modulate several culture parameters using bioreactor technologies and we will offer a perspective about the implications of the development of engi- neered niche systems both to advance scientific understanding of stem cell function and to support clinical applications.

State of the art in HSC culture systems

In the bone marrow, HSC are found both in trabecular areas close to bone surfaces (endosteal niche) and associated with the sinusoidal endothelium (vascular niche, Figure 1a) and with mesenchymal progenitors and pericytes [19e21]. HSC function is

defined as the capacity to generate all blood-cell lineages and maintain their production life-long (Figure 1b). Experimentally this is addressed by the capacity of HSC to reconstitute the hematopoietic system of lethally irradiated recipients at a clonal level. Only long-term repopulating HSC (LT-HSC) can undergo unlimited self-renewing divisions, which allow the preservation of a stem cell pool, assuring a life-long reconstitution of the blood tissue. Although a wide variety of in vitro culture conditions have been described, which promote extensive expansion of hemato-poietic progenitor cells [22,23], the maintenance in culture of LT-HSC still remains a significant challenge. In the 90s, it was shown for the first time that HSC could undergo in vitro initial clonal amplification [24,25], but with loss of self-renewal capacity, suggesting that the culture conditions were not optimal for preserving HSC features. The first HSC cultures were performed in Petri dishes on a feeder layer of stromal cells, as source of signals for HSC maintenance. Subsequently, multiple studies underlined the need for the addition of cytokine cocktails and, so far, several combinations and concentrations of soluble factors have been tested for the expansion of HSC in culture. Stem cell factor (SCF), flt-3, thrombopoietin (TPO) and interleukin-11 (IL-11), although not very efficient alone, have been shown to act synergistically to induce proliferation and to enhance survival of primitive HSC in vitro (reviewed in 24). The use of some cytokines, like IL-3, appears to be controversial, having a negative effect on HSC culture in some studies [26,27], but not in others [28,29] and, although many other growth factors, like IL-6, M-CSF, G-CSF have been introduced, consensus on a suitable medium composition is still to be reached. Indeed, a simple cocktail of soluble factors, which clearly does not recapitulate the complex microenviron- ment of the niche, may not be sufficient to provide the necessary cues to maintain and expand LT-HSC.

In an attempt to improve HSC culture models, bioreactor-based systems have been employed, introducing dynamic culture condi- tions, which may reduce mass transport limitations and optimize cell-to-cell interactions. Several types of devices, including fixed bed [30,31] and stirred suspension bioreactors [32], rotating wall vessels [33] and perfusion chambers [34,35] have been shown to modulate HSC growth, differentiation and cytokine receptor expression, and to preserve their function [36,37]. Although one of the first bioreactor based approaches eliminated the use of a stromal layer, relying only on supplementation with cytokines [38], the

expansion of human CD34þ cells could be increased in stroma-dependent systems [31,34]. Importantly, in those models only the expansion of more differentiated hematopoietic precur- sors was achieved, without establishing a physiological environ- ment that could promote LT-HSC self-renewal and prevent their differentiation or death. Moreover, these approaches were not based on HSC culture on a three-dimensional matrix, which has been proposed by several groups to efficiently sustain viability and multipotency of hematopoietic progenitors [39,40].

Recently, new high-throughput technologies, such as micro- fabricated culture platforms, have been described as useful tools to investigate the microenvironmental signals regulating stem cell fate [41]. Lutolf and colleagues showed that hydrogel-microwell arrays are a suitable system for exposing single HSC to soluble or immobilized proteins and studying how single or a combination of multiple molecules can affect HSC self-renewal and commitment [42]. This high-throughput system offers the great advantage of dissecting the role of well-defined niche signals, but does not recapitulate the complex interactions between different cell types present in the niche. It would thus seem necessary that the hypotheses generated with this approach are then tested in systems which include both cellular and acellular components, organized in a 3D environment mimicking the bone marrow stroma.

A 3D scaffold-based culture system for engineering stromal tissues

We recently reported the engineering of stromal tissues based on the 3D organization of different cell types, which are important regulators of HSC function, namely mesenchymal progenitors, osteoblasts and endothelial cells [43,44]. The models are based on cell culture within 3D porous scaffolds and require the use of a bioreactor for the perfusion of cell suspensions through the scaffold pores in alternate directions, resulting in efficient and uniform cell seeding and tissue development over several weeks (Fig. 2a) [45,46]. The perfusion system was employed for seeding and culturing freshly isolated human bone marrow nucleated cells, including both stromal and hematopoietic fractions, directly onto hydroxyapatite scaffolds. This allowed to eliminate the standard 2D expansion phase of stromal cells on plastic,

typically associated with the selection of a restricted subset of mesen- chymal populations [43]. Bone marrow stromal cells were successfully expanded within the 3D scaffolds and the resulting cellular constructs reproducibly formed bone tissue at high effi- ciency when implanted ectopically in vivo (Fig. 2b). Furthermore, the system allowed to establish a 3D co-culture of mesenchymal and hematopoietic cells, thus resembling a "stromal" tissue (Fig. 2c). When medium was supplemented with hematopoietic growth factors (e.g., SCF, interleukin-3 and platelet derived growth factor), not only mature hematopoietic cells, but also early mul- tipotent progenitors (colony forming unit-granulocyte, erythro- cyte, monocyte, megakaryocyte, CFU-GEMM) could be entrapped in the pores of the scaffold, to a higher extent than the corre- sponding 2D cultures (Fig. 2d). These results indicate that the system provided a suitable environment for the organization of stromal cells capable of supporting the survival of hematopoietic progenitors.

In addition to osteoblasts, endothelial cells of bone marrow sinusoids have been identified to play a crucial role in modulating HSC function, supporting the existence of another specialized microenvironment, the vascular niche [47]. In this context, the perfusion system described above has been also applied for the direct perfusion of human adipose derived stromal vascular frac- tion (SVF) cells, which contain a large fraction of endothelial lineage cells, through a hydroxyapatite-based scaffold. This led, after in vivo implantation in nude mice, both to extensive bone formation and to the generation of human origin blood vessels [44] (Fig. 2e), indicating the maintenance in the bioreactor-cultured constructs of vascular progenitors, preserving their differentiation potential and function. The 3D co-culture of a diverse mix of niche specialized cells was therefore achieved in this system, offering a promising perspective to start mimicking the complexity of the BM stromal compartments. An alternative experimental setup to model and investigate hematopoietic niche interactions using the previously described perfusion system is based on first establishing a stromal environ- ment as a pre-formed niche for the subsequent loading of trackable HSC (Fig. 3). This approach would allow to generate stromal tissues with defined mixtures of cell populations at selected stages of differentiation and to investigate how these parameters regulate HSC function. In a first phase, bone marrow nucleated cells were loaded and cultured for 3 weeks under perfusion onto porous, ceramic-based 3D scaffolds (Fig. 3a), resulting in a 3D cell network within the scaffold

pores (Fig. 3b). In a second phase, hematopoietic stem and precursors cells were introduced in the culture medium and perfused through the stromal tissue. After 1 week both stromal and hematopoietic cells were extracted from the scaffolds by enzymatic treatment and characterized by different techniques, including cytofluorimetry and functional in vitro and vivo assays. Cytofluorimetric analysis revealed that a population of hemato- poietic precursors was still present in culture and maintenance of HSC function was confirmed by the presence of hematopoietic colonies in vitro and spleen colonies in the lethally irradiated recipients. Transgenic strains were used to improve experimental readouts and to allow cell tracking within the scaffolds. In partic- ular, in order to minimize cell death, BM cells were harvested from Bcl-2 mice, which constitutively express an anti-apoptotic gene, whereas HSC were obtained from the bone marrow pool of GFP mice to facilitate their tracking and characterization. This simple experimental plan illustrates a possible starting point for the application of 3D scaffold-based systems for the reconstruction and modeling of specific elements of the bone marrow environment. Within the system, several parameters can be systematically varied, thus modulating the level of complexity and allowing to address specific biological questions. The following section, schematically summarized in Fig. 4, exemplifies some of these parameters and critically discusses their potential role.

Modulating parameters of the proposed 3D perfusion culture system

4.1. Scaffold properties

Several substrates, in the form of macroporous carriers, micro- spheres and nonwoven fabrics, have been tested in the past to achieve efficient HSC expansion, with various degrees of success [48,50]. More recently, scaffold-based 3D culture systems have been proposed to reproduce structural and/or functional features of the BM environment, offering a unique platform to address fundamental questions related to stem cell biology [38]. In partic- ular, controlled modifications of the scaffold, architecture, compo- sition and stiffness, which have been shown to play a critical role in

determining stem cell fate [51,52], would allow to further elucidate how specific chemico-physical cues can mimic and regulate the development of stem cell niches.
Molecular functionalization of the scaffold could also contribute in the establishment of a physiological niche environment increasing the interactions with the HSC or with different populations of the stromal tissue. Segment-1 (CS-1) and RGD motifs, which mimic the fibronectin domains of the ECM, have been shown to have an advantageous effect for the expansion of CD34þ cells [53] and specific signals could be introduced to improve cell migration (e.g., chemokine (C-X-C motif) ligand 12) and adhesion (e.g., cadherins). In this context, hydrogels represent an attractive class of materials since they offer large opportunities for functionalization and allow for the evaluation of single or multiple molecules on stem cells [54]. More- over, they can undergo fine tuning of their bulk properties, such as stiffness and shape, and, being transparent, they also allow a direct monitoring and characterization of the cells during the whole culture.
In the 3D perfusion-based culture system described above (Fig. 3), whereby a stromal tissue is first engineered as a niche prior to HSC loading, the scaffold would influence HSC fate not only by direct interaction, but also indirectly, by guiding the establishment of a specific stroma. For example, scaffolds mimicking the compo- sition or stiffness of bone tissue (e.g. ceramic-based materials) will induce an osteogenic differentiation of the seeded mesenchymal progenitors and their deposition of bone-like extracellular matrix,

possibly resulting in the establishment of a specialized osteoblastic niche. The system thus offers the opportunity and challenge to test the regulatory function of specific scaffold properties both on HSC and on their niche.

4.2. Culture conditions

Since HSC in the bone marrow have been found to be exposed to hypoxic conditions, many groups have examined the effects of Dissolved Oxygen Concentrations (DOC) on the maintenance, proliferation and differentiation of HSC during in vitro culture.

Several reports have shown that hematopoietic progenitor cells can be better maintained in vitro by keeping low levels of DOC [55,56], but the mechanisms of oxygen related aspects on HSC have not been fully elucidated yet. In fact, whether the loss of hematopoietic capacity is directly due to the oxidizing effect of high DOC [57,58] or is explicated through more complex cellecell interactions or humoral factor stimulations by niche cells [59], which are in turn influenced by oxygen levels, has not been clarified. Moreover, the general lack of monitoring and control of oxygen levels in conventional 2D model systems led to poorly characterized culture conditions. Significant oxygen gradients have been measured throughout the depth of stagnant culture media in static 2D cell cultures (i.e., O2 applied at surface of media vs measured at the cell level) [60], an effect which could be exacerbated by a highly- oxygen-consuming confluent stromal feeder layer. Perfusion of culture media over the cells (i.e., within the cell-seeded pores of a 3D scaffold), instead, could mitigate oxygen gradients and facili- tate the monitoring and control of oxygen levels in the culture via in-line sensors, and thus, support the establishment of a well- defined and controlled model system [46].

Similarly to DOC, low pH has been shown to negatively affect HSC maintenance in vitro [61]. To deepen the study on pH effects and to monitor pH changes over the whole period of culture, an approach equivalent to the one proposed for oxygen measurements could be established, based on non-invasive sensors able to continuously measure the pH during the culture. Ideally, in a bioreactor system the sensors could be interfaced with the gas supply of the culture environment in order to change the CO2 percentage to keep a constant pH value throughout the whole culture time.

High stirring speeds during in vitro culture have been described to stimulate HSC differentiation, indicating that HSC are relatively sensitive to shear stress. The lack of suitable instruments to directly measure local shear stress for HSC cultures has so far prevented the clear definition of suitable shear stress regimes. Thanks to the implementation of bioreactor technologies, recent studies revealed that shear stress can be beneficial for MSC, stimulating their proliferation and differentiation [61e64]. Investigating the ranges of suitable shear stress for both MSC and HSC when co-cultured represents a fascinating challenge that will require the introduction of

computerized fluid dynamic simulations (CFD) for defined vessel geometries and scaffold macro- and micro-pore architectures to predict local stress values [65].
According to earlier work on in vitro HSC co-cultured with stromal cells, feeding rate and medium exchange are also expected to play a role in the maintenance and proliferation of hematopoietic progenitors [66]. Frequent medium changes represented a great advantage for hematopoietic precursors cultured in static [67,68] or dynamic conditions [34], possibly because of the active removal of cell-derived negative regulators. In this regard, perfusion-based bioreactor systems, due to their configuration and functioning principle, are ideally suited for controlled tests on different medium exchange schedules. Moreover, medium refreshing can be implemented in a continuous as opposed to batch mode, in order to maintain specific culture parameters (e.g., pH) at controlled levels.

4.3. Co-culture of different cell types

It has long been known that feeder layers generated by mesen- chymal cells can support the maintenance of HSC in culture [66]. In more recent times, we have observed an increased recognition of the important role played in vivo by several cell types (e.g., osteoblasts, osteoclasts, endothelial cells and pericytes) in the generation of HSC niches. A culture setting supporting the use of different combina- tions of cell types, some of them possibly genetically modified to test the relevance of specific factors, is therefore required to allow in vitro models of a higher complexity, which more closely reproduce the in vivo environment. As compared to standard 2D culture systems, a scaffold-based model would facilitate a 3D spatial distribution of the different niche cell compartments, resulting in a structural organization which could better resemble the in vivo counterpart. Moreover, the use of a perfusion system for cell seeding would offer the opportunity of efficient and uniform distribution of the different cell populations, even if introduced at different stages of formation of the stromal tissue, when reduced construct perme- ability would otherwise confine cells in the outer periphery. In this regard, the scaffold used in the system could be designed with features (e.g., spatially differential composition or pore size) enabling to regulate the establishment of differential cellecell interactions within specific compartments.

An attractive consequence of a complete reconstruction of the bone marrow niche environment with its different cell types could be the engineering of a self-maintaining system, which does not depend on external supply of cytokines for HSC preservation. This would overcome the regulatory issues related to the use of recombinant cytokines in the perspective of a clinical application, coupled with the advantages of reducing the costs of expensive medium components.

Perspective: relevance of engineered 3D stromal tissues in different fields

In the previous sections we highlighted the potential of combining a controllable perfusion-based culture system with 3D scaffolds for the ex-vivo reconstruction of specific components of the bone marrow niche. In this section, we will provide a few examples on how the system could be exploited as a model to investigate the interactions between HSC and their regulatory environment, as well as a tool to improve stem-cell based therapies by supporting HSC maintenance. We will then conclude by out- lining possible extensions of the system towards the engineering of other stromal niches.

HSC transplantation is the only treatment by which many cases of hematopoietic malignancies can be cured. However, the amount of LT-HSC that can be transplanted is limited by the amount that can be harvested, since their extensive in vitro expansion is not currently possible without loss of their stemness [69]. In this regard, the application of the system we propose for ex-vivo expansion of LT-HSC, especially if derived from umbilical cord blood, generally associated with high quality but limited numbers, would be extremely relevant. The final goal would be to engineer both quiescent niches and microenvironments which support self- renewing divisions in order to allow LT-HSC expansion (Fig. 5a) [70,71]. Thanks to the possibility to control the cellular and molecular makeup of the engineered niches, the proposed culture system could also offer a modular platform to identify the role of specific signals and defined accessory cells (e.g. regulatory T cells, natural killer cells) which support HSC homing and engraftment (Fig. 5b). This application is clinically relevant to define strategies to improve the efficiency of HSC transplantation and to

shorten the period of post-transplant immunodeficiency by accelerating T cell reconstitution. It would be tempting to speculate that the 3D system mimicking the BM environment could also be used as an engineered bone marrow organ, to be ectopically grafted to support the function of transplanted HSC when the niche is known to be compromised (Fig. 5c).

The system may also offer the opportunity to study the role of the HSC niche in pathological conditions. In fact, recent findings raised the possibility that, as for HSC, also the survival of malignant cells is mediated by the niche environment. Acute myeloid leukemia (AML) has a hierarchical organization similar to that of normal hematopoiesis, where a small subpopulation of cancer cells, called leukemia stem cells (LSC), holds the ability to initiate the disease. LSC often display a more mature phenotype compared to normal HSCs [72], but nevertheless harbor unlimited and de-regulated self- renewal activity mediated by a series of oncogenic mutations [73e75]. Some studies have shown that LSC receive signals from the niche that support self-renewal, exploiting the mechanisms involved in the long-term maintenance of HSC them-selves [76]. The recent findings regarding the interaction between LSC and the niche open the possibility of identifying new targets for AML therapy, which is currently successful only in less than 50% of the cases [77]. These approaches should be directed to selectively stop LSC growth by interfering and disrupting their interactions with the niche environment and, at the same time, they should preserve normal hematopoiesis to ensure HSC regeneration. The proposed 3D in vitro system would offer the opportunity to dissect the mechanisms related to LSC maintenance, combining human cells with cells har- vested from different strains of transgenic mice, and would repre- sent a useful platform for drug screening (Fig. 5d, f).

The reconstruction of a functional bone marrow organization could be exploited not only as a niche for HSC, but also for mesenchymal stem cells (MSC), which are known to be conditioned by stromal factors [78]. This model would significantly contribute to clarify several mechanisms which regulate maintenance of MSC self-renewal and differentiation capacity. The resulting culture system may find application for the generation of advanced cellular grafts for bone tissue repair (Fig. 5e), as well as a model to identify and test new molecules for the pharmacological treatment of bone- related disorders (Fig. 5f).

Finally, the concept of engineering a 3D niche could be extended to other stem cell systems (Fig. 5g). For example, engineered stromal tissues would be a useful tool to address questions related to cancer stem cell biology and may allow to maintain and thus better characterize cancer stem cells in a defined in vitro setting. This would bridge the gap between 2D cultures, which do not reproduce the complexity of the in vivo environment, and animal models, which, although more physiological, do not allow to address the role of individual cell populations present at the tumor site [79]. Furthermore, the 3D spatial organization of specialized stromal cells, which has been shown to be fundamental to support the function of several stem cell systems, may also be exploited for the engineering and regeneration of tissues for which disruption of the architectural structure causes degeneration and loss of function (e.g., the thymus).

In conclusion, we propose that 3D scaffold-based culture systems, supporting the development of both cellular and acellular niche components in controlled settings, can be applied for a physiological reconstruction of the stem cell regulatory environment. The resulting models, derived by the combination of mate- rial- and tissue-engineering fields with cell and molecular biology, will have a direct impact on unraveling the mechanisms regulating stem cell function and on the development/validation of new therapeutic strategies for a variety of clinical needs.

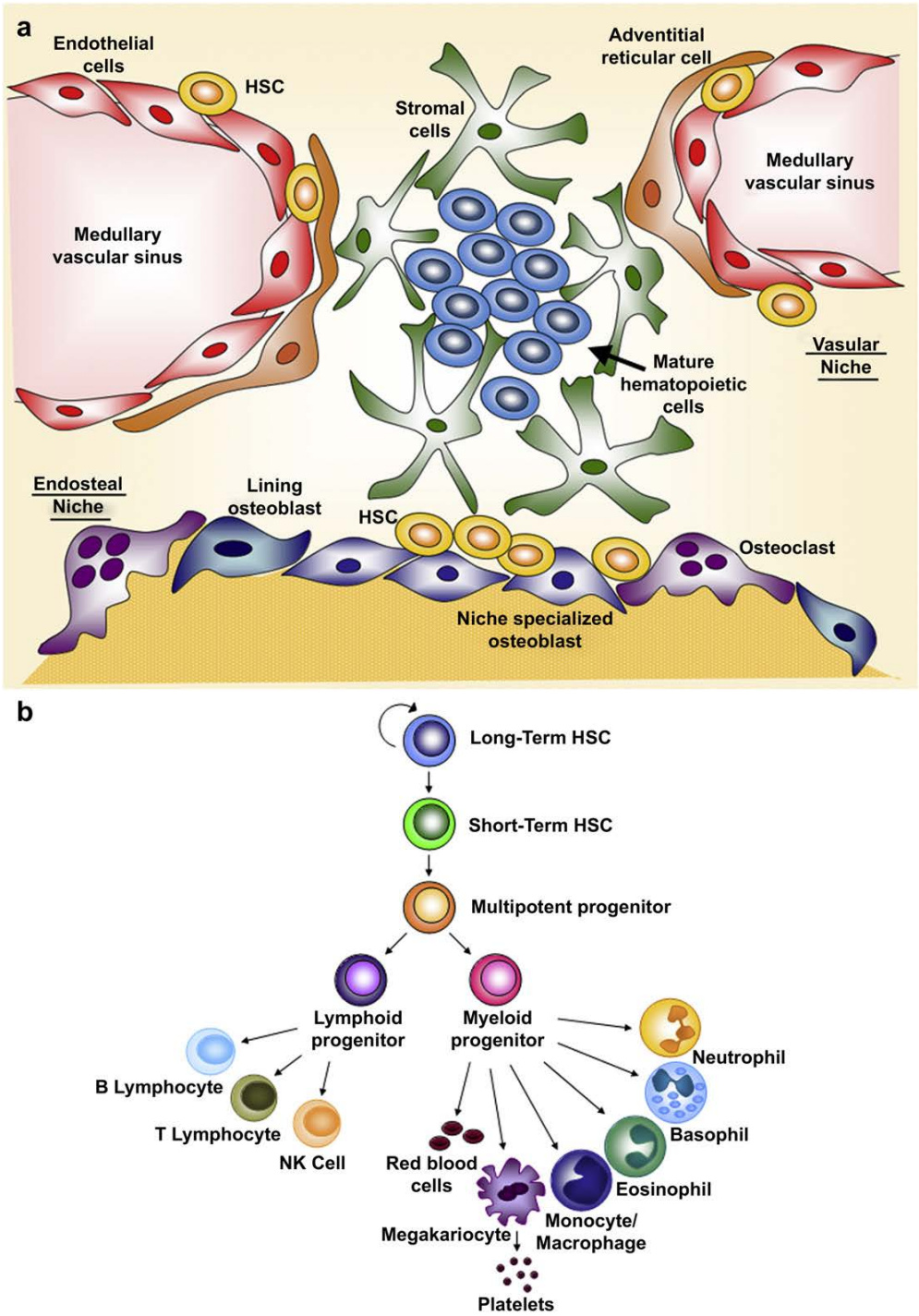

Fig. 1. a) HSC have been localized close to the endosteal surface in contact with specialized niche osteoblasts (endosteal niche) and in proximity of medullary sinuses (vascular niche). Other cell types, like osteoclasts and adventitial reticular cells/pericytes, seem also to be involved in the regulation of HCS fate. b) HSC hierarchy. Long-term HSC (LT-HSC) have the capacity to generate all the blood-cell lineages but they are also able to assure the maintenance of a stem cell pool through self-renewing divisions.

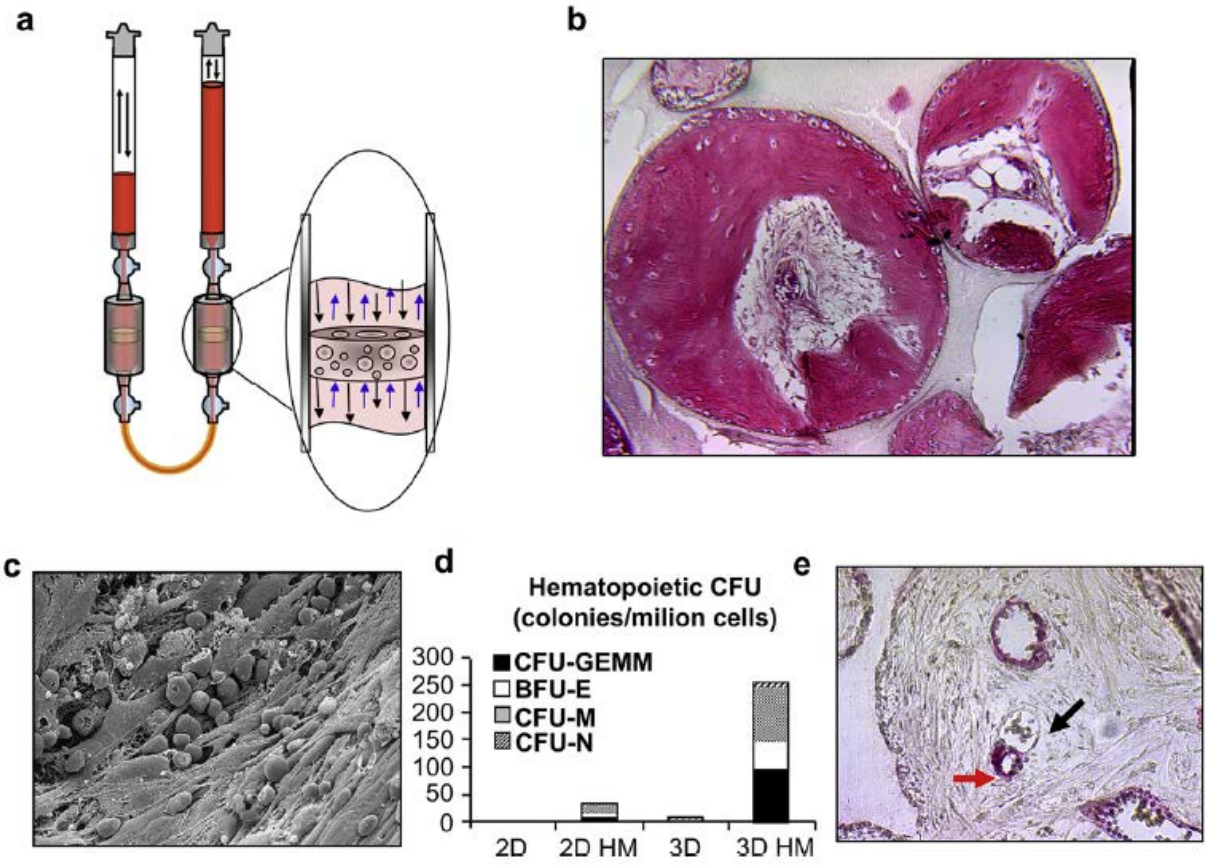

Fig. 2. a) Schematic representation of a 3D perfusion bioreactor-based culture system; where a cell suspension or culture medium are perfused directly through the scaffold pores respectively for cell seeding and prolonged 3D cell culture. b) Representative field of hematoxylin and eosin stained histological sections of constructs generated by the perfusion culture of human bone marrow nucleated cells through a ceramic scaffold for 3 weeks, followed by ectopic implantation in nude mice for 8 weeks. The strong eosin staining and the presence of osteocyte lacunae within the pores of the scaffold, characteristic of bone tissue, indicate the osteogenicity of the implanted constructs. c) Scanning electron microscopy image of a 3D stromal tissue, generated using the perfusion system described above and consisting of a 3D-network of heterogeneously shaped cells and extracellular matrix [43]. d) Quantification of the hematopoietic colony-forming units present within the populations generated after 2-dimensional (2D) or three-dimensional (3D) culture in standard or hematopoietic medium (HM), supplemented with SFC, IL-3 and PDGF. e) Representative section of constructs generated by human adipose tissue-derived cells cultured under perfusion for 5 days and implanted in nude mice for 8 weeks. The section, immunohistochemically stained using an antibody against the human isoform of CD34, demonstrates the formation of human origin (red arrow), along with murine (black arrow) blood vessels [44].

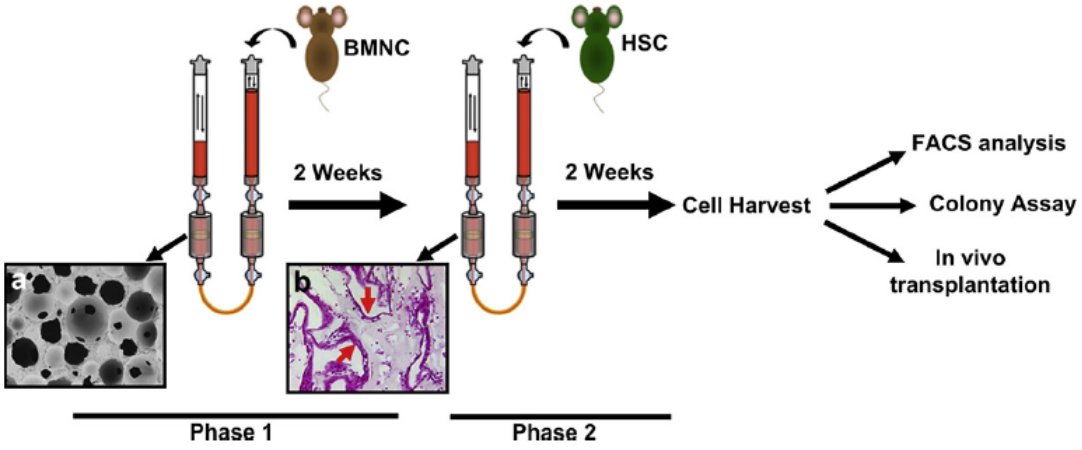

Fig. 3. Possible experimental approach for reconstructing the bone marrow niche environment in a 3D perfusion-based bioreactor system. During Phase-1, Bone Marrow Nucleated Cells (BMNC) are seeded and expanded within the pores of the scaffold (a), generating a stromal tissue (b, red arrows). HSC, harvested from GFP mice, are then loaded into the perfusion system and cultured throughout Phase-2. Cells are then harvested and characterized for their phenotype and function.

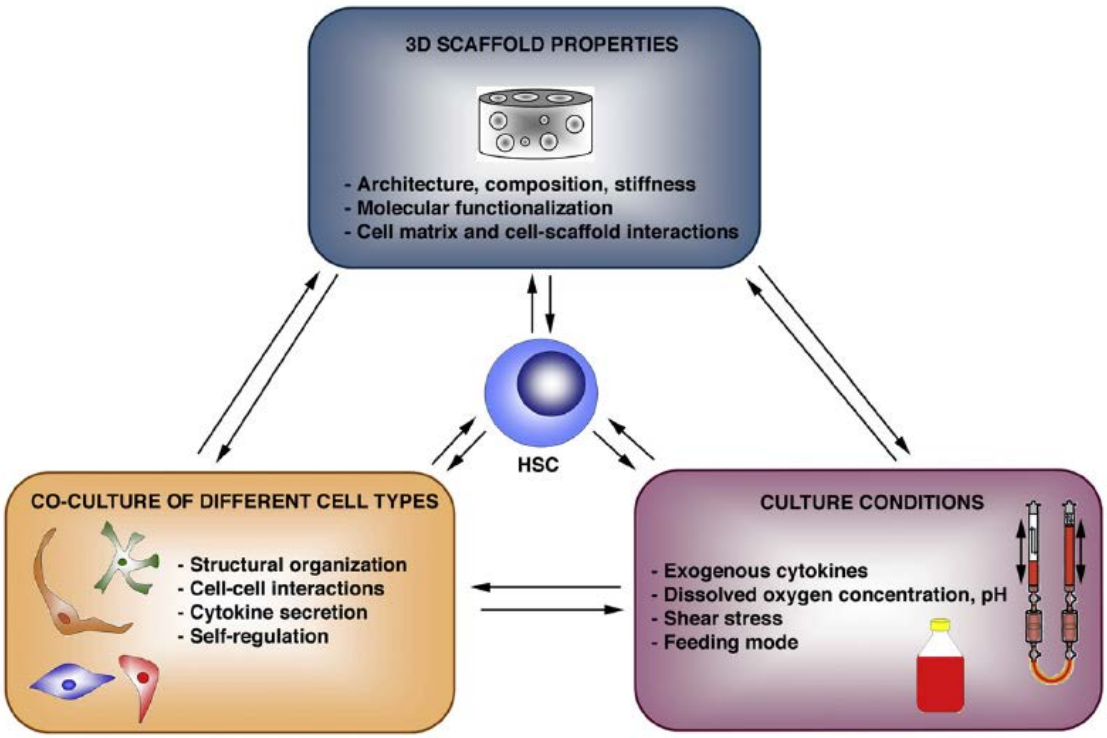

Fig. 4. Schematic illustration of the parameters that can be modulated in the described 3D perfusion-based bioreactor system to reconstruct an artificial niche and to selectively influence HSC fate. The bulk properties of the 3D Scaffold, together with a specific surface functionalization, can be adapted to resemble one or more specific aspects of the niche. The Co-Culture of Different Cell Types with the HSC is another crucial feature of the bioreactor system to recreate the physiological complexity of a stromal supportive tissue and to establish the formation of a functional niche. The high degree of flexibility and control over Culture Conditions provided by the bioreactor system is thus crucial to reproducibly constitute a suitable culture environment and to deliver to the HSC the selected cues.

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Conclusions and final remarks

Summary

This thesis work focused at validating 3D scaffold-based culture systems as potential tools to ex-vivo recapitulate at various extents the main components of native tissues. In general, it was demonstrated that the more physiological environment provided by the combination of cellular and acellular factors can positively affect the functionality and the overall cell behavior in different experimental setups. Thus, efforts have been directed towards mimicking functional features of the stromal tissues of origin of the cultured cells.

In chapter I, a new paradigm for the expansion of human bone marrow-derived MSC was proposed. Traditional cultures on 2D plastic could be completely bypassed in favor of a scaffold-based 3D approach supported by a perfusion bioreactor. Cells cultured in different conditions displayed similar surface marker expression, but a striking difference with regard to the maintenance of better progenitor features in terms of higher clonogenic potential and multilineage differentiation capacity. These features might prospectively reveal to be key to improved therapeutic activity of cultured MSC. In addition, the 3D model demonstrated its suitability to both industrial exploitation (by substituting bulky 2D flasks with compact 3D scaffolds) and research use as a stromal bone marrow environment (by allowing more reproducible results and increasing the number of tunable variables).

In chapter II, the 3D approach was challenged with adult mouse TEC, a cell type that has so far escaped any attempt to be cultured without undergoing loss of function. First, we demonstrated that freshly-isolated uncultured cells maintain a limited but consistent capacity to support thymopoiesis in vivo when implanted in conjugation with a 3D scaffold, while 2D expanded cells lose this capacity. Arguing that it was the culture step that induced this dedifferentiation, we proposed an alternative method based on the use of 3D scaffolds. Therefore, we showed that embedding in a 3D fibrin-based substrate of freshly isolated TEC was a crucial step to prevent TEC de-differentiation. In fact, if an expansion step on 2D was performed preliminarily to 3D culture, loss of function could not be rescued. Thus, the unique features of the fibrin hydrogel compared to plastic, arguably stiffness, biodegradability as a mean to cell motility, and compliance to cell adhesion in a 3D architecture demonstrated to be instrumental to preserve TEC function.

Finally, in chapter III we validated the concept described in chapter I focusing on the possibility of recapitulating the hematopoietic niche in a bioreactor-based approach supported by a ceramic scaffold. In fact, HSC can be cultured in an engineered stromal like tissue while maintaining their proliferative potential and overall hematopoietic functionality. The model represented a flexible platform suitable to tailor multiple parameters in controlled fashion, and we proposed a prospective exploitation of the system to target in vitro experimentation in a more reliable way.

Relevance of the study and future perspectives

The lack of relevant in vitro biological models able to accurately represent the behavior and the function of native tissues and organs has severely affected scientists' ability to unveil the complex intrinsic and extrinsic cues regulating cell biology.

However, interdisciplinary interactions between biologists, chemists, engineers, physicists, and clinicians have brought to a sudden acceleration in the development of alternative approaches to target a vast array of biological questions and needs, from basic science investigations to new therapeutic treatments.

The model described in chapter I is an example of how the same 3D culture systems can address both research and clinical needs: scalability, standardization, and compactness make the proposed perfusion-based system a powerful tool for manufacturers of cell-based products; at the same time the maintenance of more progenitor properties is an intriguing aspect for biologists. Remarkably, cell-based therapies represent an increasing market as evidenced by the fact that only in the first quarter of 2012, new capital investments for $85 million flowed in publicly traded companies, clinical trials continued to progress and the industry continued to show promising growth. In particular, MSC are undergoing several clinical trials to evaluate safety and efficacy for their use on the basis of their immunomodulatory properties in autoimmune diseases. Our platform proposes a method to reproducibly culture in a compact system MSC with a distinct signature when compared to cells grown in 2D; however, further investigations to assess how the expansion of MSC in a compliant in vitro environment can affect their mechanism of action and potency after in vivo transplantation are urged by the high number of patients that might benefit of more available, better-defined, and effective MSC transplant. Similarly, the bioreactor system and its ability to maintain a more heterogeneous, progenitor-like MSC population can

be a key tool for a better understanding to fill the gaps in our understanding of MSC biology.

The approach described in chapter II was proposed for a comparable purpose in a different organ, i.e. culturing in vitro a TEC-based thymic organoid to get a privileged access to the biological processes regulating thymopoiesis, the central mechanism leading to T cell mediated adaptive immunity. T cell selection is a tightly controlled process, and many efforts are currently devoted to encrypt the multicellular cross-talk between developing thymocytes and the thymic stroma. Prospectively, the optimization and the exploitation of the proper environmental conditioning provided by the 3D fibrin scaffold for the culture of functional TEC could lead to a better understanding of thymopoiesis mechanisms, and in a second instance, to the manipulation of these mechanisms to educate T cells with custom-defined specificities and functions for therapeutic applications.

It should be noted that, although failure in thymic functionality for genetic mutations or morbidity following chemo- and radiotherapy can lead to life threatening diseases, there are currently no approaches in human to boost thymic reconstitution. Hence, a tissue engineering approach directed towards thymus regeneration that allows ex vivo manipulation of TEC would be of fundamental importance. Moreover, the model could facilitate the identification of the so far unknown progenitor/stem TEC.

Finally, in chapter III we provide a bioreactor based approach able to recapitulate the cellular and biophysical aspects of the HSC niche complexity. This method is alternative and complementary to a different engineering approach, targeted to dissect the niche in its single signals (e.g. ligand presentation from the biomaterial or extracellular matrix, spatial and temporal patterned activation of receptors on the cells surface) and exerting tight control over them. We propose that investigation of the extrinsic signals

of the niche regulating stem cells self-renewal and differentiation, and implementation of technologies able to transpose into factual tools the generated knowledge, are instrumental steps to be taken to ultimately translate scientific research into clinical application. In addition, an engineered stromal niche able to mimic the bone marrow microenvironment would be an invaluable tool to investigate homing patterns of metastatic tumor cells that, upon entry the bone marrow and acquisition of a quiescent state, can escape eradication therapies thus causing rumor recurrence in a later stage. Currently, these studies require a high number of animals and are affected by limited visual access to the stroma. Hence, our in vitro 3D model could serve as a surrogate to limit animal experimentation and use more relevant human cells.

Taken together, these results highlight that an increase in the complexity of the traditional culture systems is crucial to better recapitulate the functional microenvironment of stromal and stroma-dependent cells or stem cells. Growing and handling cells in a 3D structure combined with a compliant biomaterial and bioengineering tools can dramatically increase the relevance of scientific data, enable unpredecented modalities to control the artificial microenvironment, and decrease the need of costly, time consuming, and ethically debated in vivo experiments.