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Stem cell plasticity, osteogenic differentiation and the third dimension

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Abstract Different cues present in the cellular environment control basic biological processes. A previously established 3D microwell array was used to study dimensionality-related effects on osteogenic differentiation and plasticity of marrow stromal cells. To enable long-term culture of single cells in the array a novel surface functionalization technique was developed, using the principle of subtractive micro contact printing of fibronectin and surface passivation with a triblock-copolymer. Immunohistochemical stainings showed that when cultivated in 3D microenvironments, marrow stromal cells can be maintained in the wells for up to 7 days and be induced to commit to the osteogenic lineage. In conclusion, this work shows the modification of a 3D microwell array allowing the long term study of single stem cell plasticity and fate in controlled microenvironments.

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1 Introduction

The shape and degree of cell spreading has been shown to control basic biological processes [1]. Altered cell morphology correlates with a number of changes in gene expression profiles. These include, but are not limited to, up- or down-regulation of proteins involved in proliferation, ECM production, cellular signalling or differentiation [2]. This possible relationship is therefore of great interest for tissue engineering and cell based sensors, as well as for the understanding of basic characteristics of stem cell biology.

The development of distinct cell types from multipotent precursor cells is thought to occur in a two-phase process, comprising the commitment of a cell to a specific lineage, still retaining a certain degree of plasticity allowing trans- and de-differentiation, and terminal differentiation to become a specialized functional cell [3]. Encompassing a well organized series of events the gradual process of differentiation can be followed by monitoring different marker proteins being expressed at various time points. For osteogenic differentiation these comprise early genes, such as runx2, bone-specific alkaline phosphatase and collagen1 and late genes, for example osteocalcin. Depending on the cues driving the lineage commitment, this progression retains plasticity [4] or is thought to arrest the cell in a differentiation-destined state [5].

Currently, a large set of tools enables us to study the influence of individual parameters defined by the microenvironment on cell behavior. The role of physical parameters, such as matrix rigidity and cell shape, have been studied using polyacrylamide gels with tunable rigidity [6]

and two-dimensional (2D) cell patterning [7], respectively. However, the cultivation of most cell types on planar surfaces is not optimal as it does not recapitulate the in vivo situation of most cell types [8]. For the study of how cells are affected by the dimensionality of their surroundings, i.e. how cell response differs between 2D and 3D cell culture, there is a lack of standard experimental systems. Typical 3D models include matrices of either natural polymers, such as collagen, or synthetic polymers, such as PEO based gels [9]. However, it is difficult to compare results from matrices and standard flat culture substrates. The nature of many 3D matrices gives rise to less controlled environments than flat substrates, for example there can be local variations in stiffness and no control of cell shape is possible. Therefore, a novel technique was recently developed that utilizes polydimethylsiloxane (PDMS) based microwell arrays to cultivate single cells in a 3D environment with control over cell shape, stiffness and biochemical coating [10, 11]. This experimental approach makes it possible to investigate dimensionality dependent responses independently of other parameters of the microenvironment.

The microwell array was produced by a combination of microfabrication in PDMS and protein patterning of the chip surface, limiting cell adhesion confined to the well surface area [10]. The array has been used to study dimensionality dependent actin organization and cell contractility (Ochsner et al. in preparation). However, while actin organization is a process that can be studied within hours, other biological processes require days to weeks in culture. With the currently used patterning strategy of the microwell array, which is based on micro contact printing of PLL-g-PEG and functionalization with fibronectin to prevent and facilitate cell adhesion, respectively [11], we noticed reduced pattern stability and thereby insufficient confinement of cells to the wells with time (unpublished observations). We hence developed a new method for patterning of the microwell array surface to enable cultivation of cells in microwells for longer periods of time.

Impaired pattern fidelity over time has been previously acknowledged for 2D protein patterns [12] and was shown to be influenced by both cell-dependent and -independent processes [13]. The stability of the patterns is also strongly dependent on the stability of the non-adhesive coating of the surrounding areas. Different approaches have been used to improve the stability of the non-adhesive coating, including the use of substrates that interact stronger with the adsorbed molecules [12]. Another approach has been to link the non-adhesive layer covalently to the substrate which gave stable patterns for at least 28 days when using polyacrylamide on glass [13]. However, these techniques are not compatible with the PDMS substrate and the 3D structure of the microwell array. Therefore we developed a new patterning method, enabling long term confinement of cells to the microwells. The new method uses a combination of subtractive microcontact printing (μCP) and adsorption of poly(ethylene oxide) (PEO)-containing block copolymers, Pluronic™, that self-assembles on hydrophobic surfaces and can suppress protein adsorption [14]. In a previous study, subtractive printing of fibronectin has been achieved by contacting microstructured hydrophobic PDMS with a Si-wafer. Although efficient, cell clusters adhered non-specifically outside of the microstructures [15]. Here we present a method that is expected to be more reliable as the removal of fibronectin is mediated through covalent linkage of fibronectin to glutaraldehyde and because the use of a relatively soft PDMS stamp ensures firm contact over the whole surface area. Furthermore, we coated fibronectin onto hydrophilic PDMS, which is known to result in a fibronectin coating which allows more efficient cell adhesion [16]. To demonstrate the feasibility of the microwell platform which allows tracking of single cells over time as a 3D, long-term culture system, human bone marrow stromal cells (HBMCs), which represent mesenchymal stem cells (MSCs), were cultivated in square shaped microwells. The shape was selected based on previous findings which demonstrated osteogenesis of single cells on square shaped 2D patterns [1].

The new functionalization strategy was developed to enable the study of fundamental cell biological processes, such as the nature of stem cell differentiation and stem cell plasticity. However, the cell array also shows promise as a high-throughput platform for studies of 3D cultured cells.

2 Experimental procedures

2.1 Microwell array fabrication

The arrays of microwells were fabricated in PDMS (Sylgard 184, Dow Corning, USA) as previously described [10]. Briefly, microstructures were created in SU-8 on a silicon wafer using standard photolithograpy. The structures were transferred into a PDMS master by molding PDMS onto the silicon wafer. After fluorosilanization of the PDMS master, it could be repeatedly used to create thin film PDMS replica on thin glass cover slips (Menzel-Gläser, Germany, strength 0, approx. 100 μm thickness) or glass bottom culture dishes (P35G-0-20-C, MatTek, USA) allowing high resolution imaging.

2.2 Surface functionalization

First, the PDMS microwell array was rendered hydrophilic by exposing it to air plasma at 0.8 mbar for 35 s (PDC-002, Harrick Scientific, USA). Thereafter the array was coated with either unlabeled or fluorescently labeled fibronectin (25 μg ml^{-1} in PBS for 1 h at RT) (Fig. 1). The coated

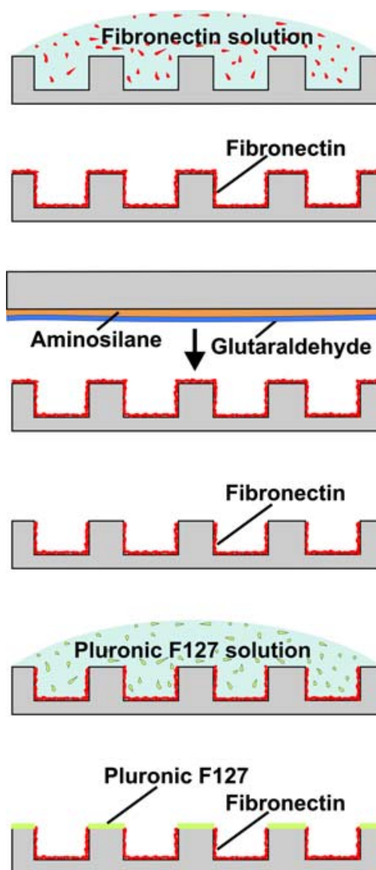

Fig. 1 Microwell functionalization schematic. The fibronectin (*red*) is removed from the chip surface by subtractive μ-CP with a glutaraldehyde (*blue*) coated stamp and the surface is subsequently passivated with Pluronic F127 (*green*). (Color figure online)

arrays were rinsed with H_2O and blown dry with N_2 just before stamping. Stamps for µCP were excised out of crosslinked PDMS sheets (1:10, curing agent to prepolymer). After plasma treatment of the PDMS stamps the surface was incubated with 2% 3-aminopropyldimethylmethoxysilane (Acros Organics, Belgium) in H_2O for 15 min. Then, the surface was rinsed with H_2O and subsequently incubated with glutaraldehyde (Sigma–Aldrich), at 0.125% in H_2O for 30 min [17]. The stamps were rinsed again with water and blown dry with N_2 before being placed in contact with the fibronectin functionalized microwell array. Complete contact over the whole area was assured by carefully pushing the stamp down with a pair of tweezers. After 30 sec the stamp was removed. The printing step was repeated 1–2 times to ensure complete removal of the fibronectin from the plateau surface. To enable hydrophobic recovery of the stamped areas the substrates were maintained for 24–48 h at RT before further functionalization. On the day of cell seeding Pluronic F-127 (Sigma–Aldrich) was adsorbed onto the substrates for 1 h, at 0.04% (w/v) in PBS. After rinsing with PBS the substrates were used for cell cultivation.

2.3 Isolation and culture expansion of HBMCs

Human bone marrow stromal cells (HBMCs) were isolated as described previously [18], with minor modifications, from femur-derived bone marrow specimens of a patient (female, age 58) undergoing hip surgery after informed consent. In brief, samples including pieces of trabecular bone were incubated in isolation medium (25 mM HEPES, 128.5 mM NaCl, 5.4 mM KCl, 5.5 mM D(+)-glucose, 51.8 mM D(+)-saccharose, 0.1% BSA) over night at 4°C before centrifugation at 110*g for 15 min. The supernatant was discarded and the pellet washed several times to rinse out cells from the bone pieces. After filtrating the cell suspension three times through a 200 µm polyethylenterephthalate (PET) mesh and subsequent centrifugation at 110*g for 15 min, the pelleted cells were resuspended in expansion medium (α-MEM supplemented with 10% FBS, 1%PSN, 1 ng/ml FGF-2) and 10^7 cells were seeded into T75 culture flasks. After 24 h the non-adherent cells were removed and medium was changed twice weekly thereafter. Cells were subcultured prior to confluence and used at passage one for experiments.

2.4 Osteogenic differentiation

HBMCs were seeded at a density of 240 cells/cm^2 in Petri dishes (P60) in either expansion or osteogenic medium (α-MEM supplemented with 10% FBS, 1% PSN, 10 nM dexamethasone, 50 mM ascorbic acid phosphate, 2 mM β-glycerophosphate and 10 nM 1,25-dihydroxyvitamine D_3) for 18 days before subculturing at the same density in either osteogenic or expansion medium for one or seven more days. Cells were fixed and permeabilised with 4% Paraformaldehyde/0.2% Triton X-100 for 8 min and stored in PBS without glucose until stained for bone specific alkaline phosphatase (bALP) (1:1000, Developmental Hybridoma Bank, B4-78), actin (phalloidin, 1:40, Molecular Probes, B607) and DAPI (4,6-Diamidino-2-phenylindole, 1:1000, Sigma–Aldrich D9542).

2.5 Cultivation of cells in 3D microwells

After surface functionalization the 3D microwells were rinsed extensively with sterile PBS which was thereafter replaced with either expansion or osteogenic medium. HBMCs were harvested and seeded at 10,000 cells per microwell array ($\sim$1 cm^2). The cells were allowed to settle for 15 minutes before washing off the non-adhering cells with the respective medium and were cultivated at 37°C/5%CO_2 for seven days. Medium was changed every 2–3 days.

3 Results and discussion

3.1 Surface functionalization

The microwell array could be successfully patterned with fibronectin present only inside the microwells (Fig. 2). The low fluorescence intensity on the plateau surface demonstrates that most fibronectin was removed from the plateau in the μCP step. The method was shown to be compatible with large surface areas. We created patterned areas of up to 4 cm^2 with retained pattern quality, and it may be possible to create even larger surface areas.

3.2 Stem cell plasticity

Immunohistochemical stainings against bALP (Fig. 3) showed that the HBMCs undergo osteogenesis when cultivated in osteogenic medium for 18 days. Subcultivation of the committed cells for seven more days in osteogenic medium did not alter the expression level of bALP, however, the cells subcultured in expansion medium did not stain for bALP anymore. The de-differentiation of the committed HBMCs showed that the cells retain plasticity for at least up to 18 days in culture and could be also observed when cultivating for shorter (10 or 14 days) or longer (20 or 28 days) period of times (data not shown). This is in good agreement with a previous study [4] showing that committed MSC can de-differentiate even after 30 days in culture.

The approximate total cell number was calculated from 10 random fields of view per condition and at day one after subcultivation there was no difference in cell number (112 cells in osteogenic, 106 cells in expansion medium). After seven days, the cells in expansion medium outnumbered the cells in osteogenic medium, however only by about 25% (260 cells in osteogenic, 331 cells in expansion medium). This increase in cell number in expansion medium indicates that the loss of ALP staining is not due to dilution caused by increased proliferation rates of subsets of cells and suggests that cells are actually redirected from lineage progression to increased self renewal.

3.3 Osteogenic differentiation of single cells in 3D microwells

HBMCs were cultivated in square shaped microwells, a shape shown to promote osteogenesis of single cells on 2D patterns [1]. HBMCs retain the cell shape given by the well for up to seven days in culture, which could be observed by staining for actin filaments (Fig. 4). The improved pattern fidelity over time, obtained with the new patterning method, can probably not be explained by a single factor. Instead we hypothesize that both the interaction between the PEO-based non-adhesive layer with the PDMS as well as the hydrophobic nature of the underlying substrate play relevant roles. Hydrophobic PDMS is known not to support adhesion of several cell types [19] making defects in the non-adhesive layer less problematic.

As individual cells can vary in volume, not every cell completely filled the wells. We further observed that single as well as multiple cells can bridge between functionalized wells which was due to the close proximity (20 μm edge to edge distance) of the individual microwells, as well as due to having small clusters of cells during the initial cell seeding procedure. Increasing the spacing between the individual wells however completely abolishes the unwanted bridging of cells (unpublished observations).

When cultivated within the square shaped microwells for seven days, single isolated cells stained positive for bone-specific alkaline phosphatase only when provided with osteogenic stimuli (Fig. 4). No staining for bALP could be observed when these stimuli were absent from the medium. Single HBMCs can therefore commit to the osteogenic lineage and are not hampered by the three-dimensional nature of the microwells when cultivated in osteogenic medium.

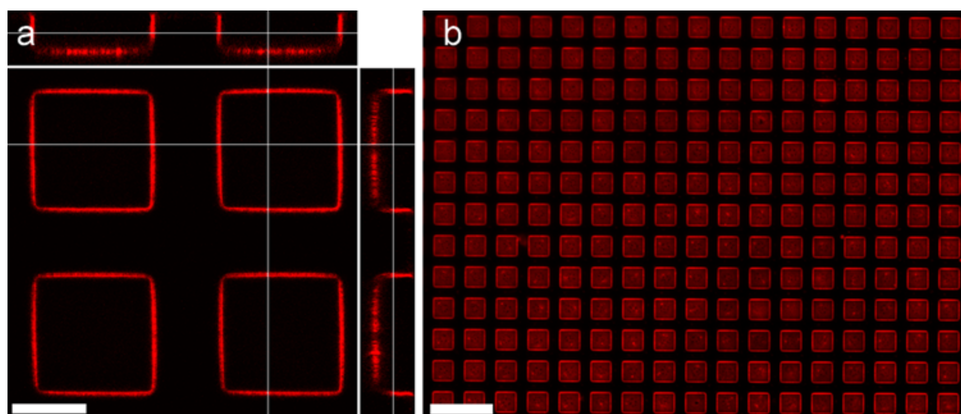

Fig. 2 Square shaped microwells in PDMS. **a** High magnification CLSM orthoslice image and **b** low magnification overview image of microwells functionalised with fibronectin (*red*) [Scale bars 20 and 100 μm, respectively]. (Color figure online)

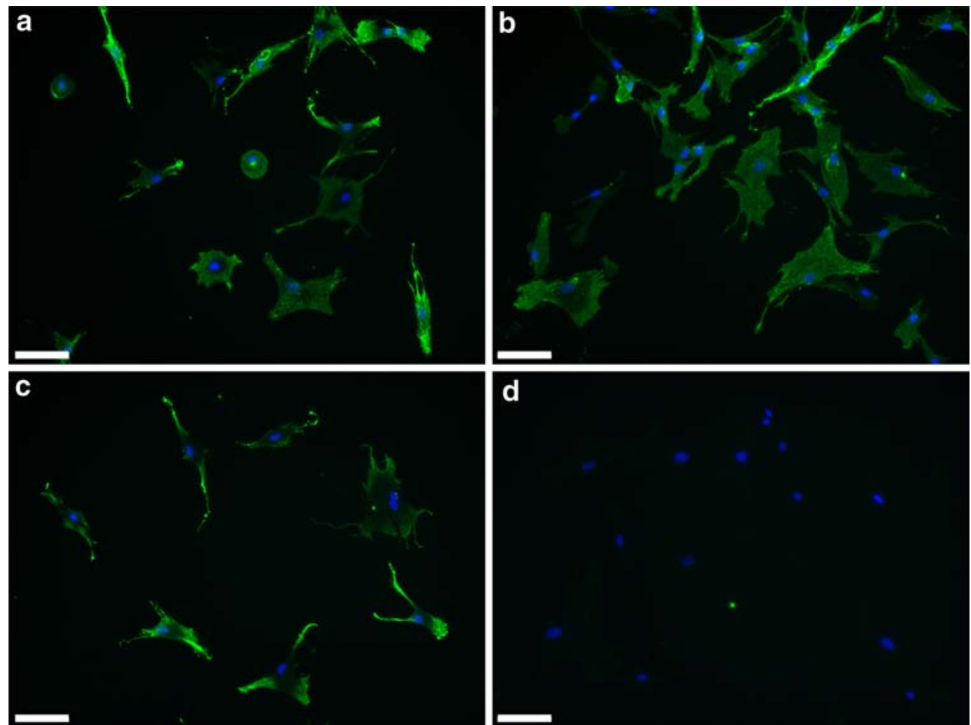

Fig. 3 Stem cell plasticity. HBMCs cultivated for 18 days in osteogenic medium and subcultivated for 1 (**a**, **c**) and 7 (**b**, **d**) days in osteogenic (**a**, **b**) or expansion (**c**, **d**) medium. The cells were stained for bALP (*green*) and the nucleus (*blue*) [Scale bar 100 µm]. (Color figure online)

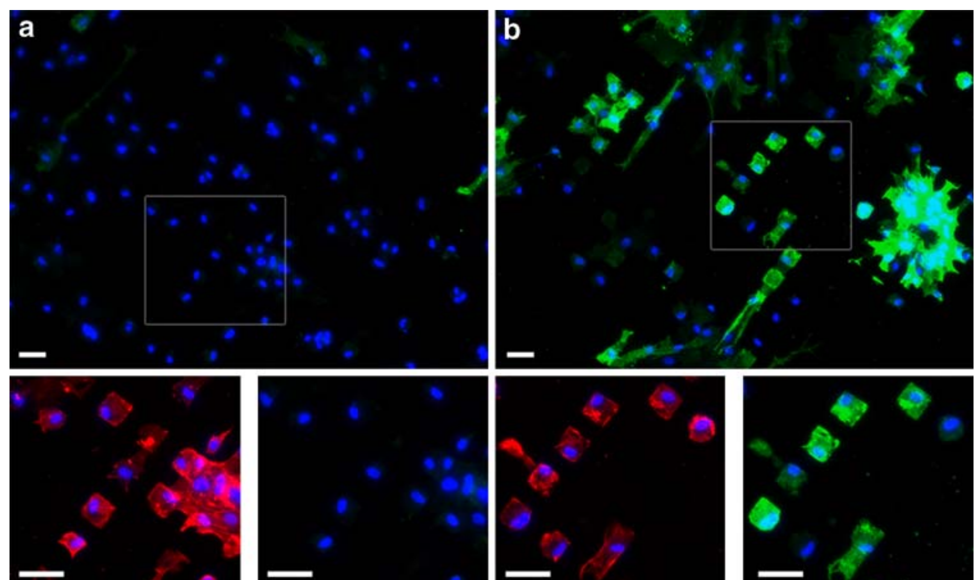

Fig. 4 Osteogenic differentiation of single stem cells in microwells. HBMCs cultivated for 7 days in square shaped microwells in expansion (**a**) or osteogenic (**b**) medium and subsequently stained for bALP (*green*, large and small right), actin (*red*, small left) and the nucleus (*blue*) [Scale bar 50 µm]. (Color figure online)

4 Conclusion

Primary results show that the novel surface functionalization method for the microwell array allows long term cultivation of single isolated cells. Initial results show good confinement of the cells. However, cells bridging between wells and cell clustering was observed as was also described in previous studies [15]. Increasing the edge to edge distance between individual wells however completely abolished the bridging of cells. The novel functionalization method thus greatly improves the fidelity of the microwell array and is therefore efficient to study the effect of physical cues, such as dimensionality, mechanical properties of the environment, cell size and cell shape, compared to chemical cues on stem cell plasticity and lineage commitment.

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