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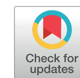
Does soft really matter? Differentiation of induced pluripotent stem cells into mesenchymal stromal cells is not influenced by soft hydrogels



Roman Goetzke [a], Julia Franzen [a], Alina Ostrowska [a], Michael Vogt [b], Andreas Blaeser [c], Gerd Klein [d], Björn Rath [e], Horst Fischer [c], Martin Zenke [a, f], Wolfgang Wagner [a, f, *]

[a] Helmholtz Institute for Biomedical Engineering, Stem Cell Biology and Cellular Engineering, RWTH Aachen University Medical School, Aachen, Germany
[b] Interdisciplinary Center for Clinical Research IZKF Aachen, RWTH Aachen, University Medical School, Aachen, Germany
[c] Department of Dental Materials and Biomaterials Research, RWTH Aachen University Hospital, Aachen, Germany
[d] Center for Medical Research, Department of Medicine II, University of Tübingen, Tübingen, Germany
[e] Department of Orthopedics, RWTH Aachen University Medical School, Aachen, Germany
[f] Institute for Biomedical Engineering – Cell Biology, RWTH Aachen University Medical School, Aachen, Germany

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ABSTRACT

Induced pluripotent stem cells (iPSCs) can be differentiated toward mesenchymal stromal cells (MSCs), but this transition remains incomplete. It has been suggested that matrix elasticity directs cell-fate decisions. Therefore, we followed the hypothesis that differentiation of primary MSCs and generation of iPSC-derived MSCs (iMSCs) is supported by a soft matrix of human platelet lysate (hPL-gel). We demonstrate that this fibrin-based hydrogel supports growth of primary MSCs with pronounced deposition of extracellular matrix, albeit it hardly impacts on gene expression profiles or *in vitro* differentiation of MSCs. Furthermore, iPSCs can be effectively differentiated toward MSC-like cells on the hydrogel. Unexpectedly, this complex differentiation process is not affected by the substrate: iMSCs generated on tissue culture plastic (TCP) or hPL-gel have the same morphology, immunophenotype, differentiation potential, and gene expression profiles. Moreover, global DNA methylation patterns are essentially identical in iMSCs generated on TCP or hPL-gel, indicating that they are epigenetically alike. Taken together, hPL-gel provides a powerful matrix that supports growth and differentiation of primary MSCs and iMSCs – but this soft hydrogel does not impact on lineage-specific differentiation.

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1. Introduction

Mesenchymal stromal cells (MSCs) comprise a multipotent subpopulation capable of differentiation into various mesodermal lineages [1]. They are used in many clinical trials – despite several limitations such as: heterogeneity of different subsets [2], lack of reliable markers for the multipotent subset [3], high variation between different donors [4], differences in MSCs from different anatomic locations [5], and drastic changes in the course of culture expansion [6,7]. At least some of these limitations may be overcome by derivation of MSCs from induced pluripotent stem cells (iPSCs) [8]. iPSCs functionally resemble a ground state of pluripotency. Directed differentiation of iPSCs toward MSCs (iMSCs) may therefore provide more homogeneous and standardized cellular products [8].

Various protocols for differentiation of iPSCs toward MSCs have been described [9,10]. To this end, we have elaborated a one-step protocol based on culture medium used for the culture of primary MSCs [8]. This medium is supplemented with 10% human platelet lysate (hPL): a non-xenogeneic substitute for fetal calf serum (FCS) that is manufactured from thrombocyte units [11,12]. iMSCs that were generated with this culture medium closely resembled primary MSCs in morphology, immunophenotype, differentiation potential, and gene expression profiles. However, with regard to DNA methylation (DNAm) profiles there were marked differences between primary MSCs and iMSCs.

Cellular differentiation is ultimately determined by epigenetic changes - and conversely epigenetic profiling might be the most

* Corresponding author. Helmholtz-Institute for Biomedical Engineering; Stem Cell Biology and Cellular Engineering; RWTH Aachen University Medical School; Pauwelsstrasse 20; 52074 Aachen; Germany.
E-mail address: wwagner@ukaachen.de (W. Wagner).

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elegant way for molecular definition of specific cell types. DNAm is concurrently the best understood type of epigenetic modification [13]: Cytosine guanine dinucleotides (CpGs) can be methylated at cytosine moieties and this governs chromatin structure and gene regulation. The DNAm levels can be precisely quantified at single base resolution and changes occur in a highly reproducible manner during development, replicative senescence and aging [3,13,14]. It has been demonstrated that epigenetic features specific for cell type, tissue of origin, senescence, and aging are largely erased upon reprogramming into iPSCs [8,15]. The epigenetic discrepancy of primary MSCs and iPSC-derived MSCs can therefore be attributed to the fact that such patterns are not fully recapitulated with the established culture conditions for generation of iMSCs [8]. To further improve lineage-specific differentiation toward MSCs it may be necessary to include additional instructive stimuli provided by supportive biomaterials.

So far, differentiation of iPSCs toward MSCs was performed on conventional tissue culture plastic (TCP) - a very stiff substrate (≥1 GPa) [16], which confines cell growth to two dimensions with flattened morphology. In contrast, the physiologic environment of MSCs consists of a 3D organized extracellular matrix (ECM) with higher elasticity [17]. In fact, it is commonly accepted that elasticity guides multilineage differentiation potential of MSCs [18,19] and that it influences differentiation of pluripotent stem cells [20,21]. Therefore, we reasoned that differentiation on such soft matrices may facilitate generation of iMSCs that more closely resemble primary MSCs.

Human platelet lysate (hPL) can not only be used as supplement for cell culture media – if hPL is combined with culture media in the absence of anticoagulants it forms a hydrogel [22–24]. These hPL-hydrogels consist of a fibrin-network and possess an extremely low elastic modulus [22]. Fibrin-based scaffolds are widely used in tissue engineering because of unique biological and mechanical properties of the polymers [25,26]. We demonstrated that hPL-gel significantly enhances growth of primary MSCs compared to normal TCP. This effect is probably due to the high concentrations of growth factors and integrin-binding sites, or to the possibility of multilayered growth at the interface between hPL-gel and the over-layered culture medium [22]. Notably, hPL-gel consists of the same components as the over-layered medium - apart from heparin - and thus the cells can be cultured on a matrix without immediate contact to additional artificial biomaterials [22]. In this study, we followed the hypothesis that differentiation of iPSCs toward MSCs is enhanced by hPL-hydrogels.

2. Materials and methods

2.1. Culture medium and hPL-gel

Culture medium for MSCs (hPL-medium) consisted of Dulbecco's modified Eagle's medium-Low Glucose (DMEM-LG) with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin (all Gibco/Thermo Fisher Scientific, Darmstadt, Germany), 10% hPL and 5 U/ml heparin (Rotexmedica, Trittau, Germany) to prevent coagulation. hPL was generated from thrombocyte apheresis units (after expiration, five days after harvesting; kindly provided by the Institute for Transfusion Medicine at the RWTH Aachen University Medical School) as described in detail before [27]. Rheological measurements of hPL-gel (three independent hPL-pools) were performed to better describe its mechanical characteristics. Cell culture experiments of this study were performed with the same hPL-pool consisting of lysates from 9 different healthy donors. In the absence of heparin, addition of hPL to culture medium resulted in hPL-gel formation as described before (hPL-gel without thrombin) [22]. To further enhance gelation – particularly to trap cells within hPL-gel – we used 0.5 U/ml thrombin (TN; Sigma Aldrich, Hamburg, Germany). Cells have been retrieved from the gel matrix with trypsin-EDTA 0.25% (Gibco/Thermo Fisher Scientific). In some experiments, we have alternatively tested a fibrin-depleted culture medium that does not require addition of anticoagulants (hPL-serum): after hPL-gel formation the hydrogel was mechanically disrupted with serological pipette and centrifugation at 2600×g for 5 min. The supernatant was then used as culture medium without additional heparin.

2.2. Rheological measurements

Rheological properties of hPL-gel were analyzed with a rheometer (Kinexus ultra+, Malvern Instruments, Worcestershire, UK) equipped with a 4° cone geometry. Shear moduli and phase angles were analyzed at 37 °C in either fluid hPL-medium or in hPL-gel upon gelation (with and without thrombin). To this end, 1.2 ml were suspended on the platform and controlled amplitude sweeps were performed at 1 Hz from 1 to 100% strain [28]. Data represent measurements of three independent hPL-pools.

2.3. Isolation of primary MSCs

Mesenchymal stromal cells were isolated from bone marrow (BM) after patient's written consent using guidelines approved by the Ethics Committee on the Use of Human Subjects at the University of Aachen (Permit number EK128/09) as described before [29]. MSCs used in this study were between passage 2 and 5.

2.4. Generation of iMSCs

Induced pluripotent stem cells were reprogrammed from the same three MSC donors that were used for experiments with primary MSCs (n = 3). Reprogramming was performed with episomal plasmids [30]. iPSCs were cultured on TCP coated with vitronectin (0.5 μg/cm^2) in StemMACS iPS-Brew XF (all Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Pluripotency was validated by *in vitro* differentiation and Epi-Pluri-Score (Cygenia GmbH, Aachen, Germany) [31]. iPSCs were re-differentiated toward iMSCs under culture conditions as indicated in the text for 35 days [8]. To this end, medium was switched from iPS-Brew XF (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to hPL containing culture conditions when iPSC colonies reached 60–70% confluency. After one week, differentiated iPSCs were maintained on 0.1% gelatine-coated TCP or hPL-gel and passaged every further week using trypsin-EDTA 0.25% (Gibco/Thermo Fisher Scientific). All experiments were performed with the three independent iPSC lines.

2.5. Proliferation analysis

Cell proliferation was analyzed after five days with AlamarBlue cell viability reagent (Life Technologies GmbH, Darmstadt, Germany) in 96-well plates (1000 cells/well; with three technical replica for each sample). Fluorescence intensity was measured after 4 h at 590 nm using a Tecan Infinite 200 plate reader (Tecan Group Ltd., Männedorf, Switzerland). Alternatively, cells were counted in a Neubauer counting chamber and cumulative population doublings were determined as described before [32].

2.6. Live/dead staining and immunophenotype of MSCs and iMSCs

Live/dead staining was performed with 10 μg/ml fluorescein diacetate (Sigma Aldrich) and 20 μg/ml propidium iodide (Life Technologies) after 7 days of culture and analyzed with an EVOS fluorescence microscope (Life Technologies). Immunophenotypic

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