EXTRACELLULAR MATRIX FROM HIPSC-MESENCHYMAL PROGENITORS MODULATES THE THREE-LINEAGE DIFFERENTIATION OF HUMAN BONE MARROW STROMAL CELLS

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Abstract

Mesenchymal stromal cells from the bone marrow (BMSCs) exhibit a functional decline during aging. We previously found that extracellular matrix (ECM) engineered from human induced pluripotent stem cell-derived mesenchymal progenitors enhances the osteogenic capacity of BMSCs. In the current study, we investigated how this ECM affects the three-lineage differentiation and secretory activity of BMSCs. BMSCs were seeded on the ECM layer and osteogenic, adipogenic and chondrogenic lineages were induced in monolayer or micromass cultures. Differentiation responses were compared to controls on tissue culture plastic after 21 days, and secretion of interleukin 6 was evaluated after 3 and 21 days of culture. We found a significant increase in BMSC growth on the ECM in all three differentiation media compared with controls. Osteogenic cultures on the ECM resulted in significantly higher alkaline phosphatase activity, osteogenic gene expression, collagen deposition, and matrix mineralization. In adipogenic cultures, a significant decline in adipocyte formation was found on the ECM. Chondrogenic induction on the ECM resulted in significantly increased chondrogenic gene expression, glycosaminoglycans deposition and collagen type II deposition, and no significant increase in collagen type X gene expression compared to control. Secretion of interleukin 6 was modulated by the three differentiation media and culture surface, and was reduced after 21 days of osteogenic and chondrogenic induction on the ECM. Together, our data suggest that engineered ECM modulates BMSCs trilineage differentiation toward enhanced osteogenesis and chondrogenesis, and reduced adipogenesis. Thus, our ECM might provide a bioactive component for enhancing osteochondral regeneration in older patients.

Keywords: Extracellular matrix, iPSCs, bone marrow stromal cells, osteogenic, chondrogenic, adipogenic differentiation.

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Introduction

Mesenchymal stromal cells (MSCs) are extensively studied for their therapeutic potential in tissue regeneration (Dominici et al., 2006; Krampera and Le Blanc, 2021). Among various adult and perinatal tissue sources of MSCs, bone marrow is one of the most commonly used and best characterized sources for bone regeneration (Marolt Presen et al., 2019). Bone marrow-derived MSCs (BMSCs) were shown to self-renew, give rise to bone, bone marrow stroma, cartilage, adipose tissue and other mesodermal lineages, and to aid in tissue regeneration via their secretory activity (Bianco et al., 2006; Kuznetsov et al., 2019; Murphy et al., 2013; Pittenger et al., 1999; Sacchetti et al., 2007).

Therapeutic use of MSCs requires a sufficient number of cells with adequate regenerative potential. It is known that MSC origin (Frobel et al., 2014; Kern et al., 2006; Lassalet et al., 2011; Mensing et al., 2011; Vidal et al., 2006, 2007), chronological age of the donor and extended in vitro cultivation can affect the number and functional properties of MSCs (Bruder et al., 1997; Fernandez-Rebollo et al., 2020; Stolzing et al., 2008; Wagner et al., 2009; Zupan et al., 2021). Various “biomimetic” strategies have been investigated to enhance MSC functionality, including in vitro culture on protein substrates and in three-dimensional (3D) biomaterial scaffolds as well as application of biochemical and biophysical signals with recognized roles in cell differentiation and tissue development (Freytes et al., 2009).
In native tissues, MSCs are surrounded by an abundant extracellular matrix (ECM), a complex network of proteins, proteoglycans and glycosaminoglycans (Arnott et al., 2011; Assunção et al., 2020; Coleman et al., 2013; Prewitz et al., 2013; Unsicker et al., 2013). The ECM provides structural support to the cells and acts as a reservoir and regulator of growth factor activity in a tissue-specific manner (Gresham et al., 2020; McKee et al., 2019; Yong et al., 2020). ECM regulates cell migration, proliferation, differentiation and autophagy, and influences stem cell fate and immunological responses (Fitzpatrick and McDevitt, 2015; Hynes and Naba, 2012; Rozario and DeSimone, 2010).

In order to mimic this complex microenvironment in vitro and enhance the number and functionality of MSCs, ECM has been engineered in various formats, including thin layers on cell culture dishes, coatings of 3D scaffolds and scaffolds composed entirely of cell-derived ECM (Ang et al., 2014; Lai et al., 2010; Lin et al., 2012; Lu et al., 2011; Tang et al., 2014; Zhang et al., 2016). ECM layers generated from human and mouse BMSCs were shown to enhance MSC proliferation and maintain their colony forming- and differentiation potentials (Lai et al., 2010; Lin et al., 2012; Sun et al., 2011). ECM layers generated from human fetal bone marrow MSCs, human adult bone marrow MSCs and human neonatal dermal fibroblasts enhanced MSC osteogenic and chondrogenic differentiation compared with standard tissue culture plastic (Lin et al., 2012; Ng et al., 2014; Pei et al., 2011), as well as maintained the chondrogenic phenotype of cultured chondrocytes (Yang et al., 2018). Rabbit BMSC-derived ECM scaffolds enhanced the thickness and homogeneity of cartilaginous tissue engineered in vitro from autologous rabbit chondrocytes (Tang et al., 2013). It was also shown that ECM supports adipogenic differentiation of MSCs (Ang et al., 2014; Mariman and Wang, 2010; Narayanan et al., 2018). In particular, ECM generated from BMSCs in adipogenic medium under macromolecular crowding conditions strongly enhanced subsequent BMSC differentiation into the adipogenic lineage compared with a standard differentiation model on tissue culture plastic (Ang et al., 2014).

Unlike primary cell sources used in previous studies, human induced pluripotent stem cells (hiPSCs) represent an unlimited source of youthful human MSC-like progenitors (hiPSC-MPs) for ECM engineering (Frobel et al., 2014; Lapasset et al., 2011; Lian et al., 2010; de Peppo et al., 2013; Sheyn et al., 2016). hiPSC-based production of progenitor cells and tissue components can be standardized and scaled up for research studies and eventual clinical translation (Frobel et al., 2014; Lian et al., 2010; de Peppo et al., 2013). In our previous study, we found that ECM engineered from hiPSC-MPs enhanced osteogenic differentiation of young adult and aged BMSCs compared to tissue culture plastic and single protein substrate coatings (Hanetseder et al., 2023). In the current study, we aimed to evaluate whether cultivation of BMSCs on hiPSC-MP-derived ECM influences not only osteogenic but also adipogenic and chondrogenic differentiation. We hypothesized that culturing on hiPSC-MP-ECM will shift the balance of trilineage differentiation and affect the secretory activity of differentiating BMSCs in comparison to BMSCs cultured on tissue culture plastic.

Materials and Methods

Materials

All materials and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated.

Cell Culture

hiPSC-MPs were generated and characterized in detail for the expression of MSC-related surface antigens and differentiation potential in our previous study (de Peppo et al., 2013). The cells were seeded on standard tissue culture flasks pre-coated with 0.1 % gelatin solution and expanded in KnockOut Dulbecco’s modified Eagle’s medium (KODMEM), supplemented with 20 % HyClone fetal bovine serum (FBS), 2 mM GlutaMAX, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol (all from Fisher Scientific, Pittsburgh, PA, USA), 100 U/mL penicillin-streptomycin (P/S) and 1 ng/mL basic fibroblast growth factor (b-FGF) (Invitrogen, Fisher Scientific). The cells were cultured in a humidified incubator at 37 °C and 5 % CO2 with media changes twice per week until confluent.

Bone marrow mononuclear cells of a 20-year-old female donor were purchased from Lonza (Basel, Switzerland) and seeded on tissue culture plates. Cells from the same young adult donor were used in our prior study (Hanetseder et al., 2023). Non-adherent cells were removed with media changes and adherent cells, i.e., BMSCs, were expanded in medium consisting of Dulbecco’s modified Eagle’s medium high glucose (DMEM-HG), supplemented with 10 % FBS, 100 U/mL penicillin-streptomycin (P/S) and 2 mM L-glutamine and 1 ng/mL b-FGF (PeproTech®, Fisher Scientific) with regular media changes twice per week. Upon confluence, BMSCs were passaged and characterized to confirm the expression of MSC surface antigens and the trilineage differentiation potential according to the consensus position statement by the International Society for Cellular Therapy (Dominici et al., 2006) (data not shown). Cells of passage 5 were used in the following experiments.

Generation of hiPSC-MP-ECM Layer

hiPSC-MP-ECM layer was generated and decellularized as described in our previous study (Hanetseder et al., 2023). Briefly, cells were seeded on 24-well plates at 19,000 cells/well and cultured in ECM medium consisting of DMEM-HG, 10 % FBS, 100 U/mL P/S, 2 mM L-glutamine and 50 µM ascorbic acid-2-phosphate (A2P) for ten days, when the formation of a dense ECM layer was confirmed. ECM was washed with phosphate-buffered saline (PBS) without Ca2+ and Mg2+ (Lonza) and decellularized as described in our previous study (Hanetseder et al., 2023). Briefly, cells were seeded on 24-well plates at 19,000 cells/well and cultured in ECM medium consisting of DMEM-HG, 10 % FBS, 100 U/mL P/S, 2 mM L-glutamine and 50 µM ascorbic acid-2-phosphate (A2P) for ten days, when the formation of a dense ECM layer was confirmed. ECM was washed with phosphate-buffered saline (PBS) without Ca2+ and Mg2+ (Lonza) and decellularized as described in our previous study (Hanetseder et al., 2023).
larized by incubation in 0.5% Triton X-100 buffer containing 20 mM NH₄OH in PBS for 15 min at 37 °C. After washing with PBS, ECM was incubated in 100 U/mL DNase I solution in DPBS for 1 h. Decellularized ECM was washed and stored until use in PBS containing 100 U/mL P/S at 4 °C.

**Trilineage Differentiation of BMSCs on the ECM Layer**

All differentiation experiments were conducted using the same control medium consisting of DMEM-HG with 10% FBS, 2 mM L-glutamine and 100 U/mL P/S or a specific differentiation medium.

To induce osteogenic differentiation, BMSCs were seeded at a density of 5000 cells/cm² on tissue culture plastic or ECM layer in 24-well plates, and cultured for up to 42 days in either control medium or osteogenic differentiation medium consisting of control medium further supplemented with 10 nM dexamethasone, 50 µM ascorbic acid 2-phosphate (A2P) and 10 mM β-glycerophosphate.

To induce adipogenic differentiation, BMSCs were seeded at a density of 7400 cells/cm² on tissue culture plastic or ECM layer in 24-well plates, and cultured for up to 21 days in adipogenic differentiation medium consisting of control medium further supplemented with 0.5 mM 3-Isobutyl-1-methylxanthine (Serva, Heidelberg, Germany), 60 µM indomethacin, 0.5 µM hydrocortisone and 1 µM dexamethasone. Higher cell density in the adipogenic group was used as an additional stimulus for adipogenic differentiation. For the adipogenic differentiation control groups, the cells were seeded at a density of 5000 cells/cm² on both surfaces and cultured in control medium.

To induce chondrogenic differentiation, BMSCs were seeded as cell micromasses consisting of 100,000 cells/20 µL droplet of control medium on tissue culture plastic or ECM layer in 24-well plates. The micromasses were allowed to adhere for 1 h before adding culture medium. The micromasses were cultured for up to 21 days in either control medium or chondrogenic differentiation medium consisting of DMEM-HG supplemented with 40 µg/mL L-proline, 100 nM dexamethasone, 50 µg/mL A2P, 1% insulin-transferrin-selenium-ethanolamine (ITS-X mix 100x), 0.91 mM sodium pyruvate and 1% P/S (all from Thermo Fisher) and 10 ng/mL human transforming growth factor-β3 (TGF-β3) HumanKine®.

**DNA Content Quantification**

For DNA content quantification, the BMSC cultures were washed with PBS and incubated in a digestion buffer containing 150 mM NaCl, 55 mM Na Citrate, 20 mM EDTA, 0.2 M NaH₂PO₄ (Honeywell Research Chemicals, Morris Plains, NJ, USA), 10 mM EDTA, 6 U/mL papain, and 10 mM cysteine in dH₂O (with pH 6.0) overnight at 60 °C. The digested samples were collected, centrifuged for 5 min at 300 × g, and DNA content of the supernatant was determined using Hoechst 33342 dye. 100 µL of Hoechst working solution consisting of 5 µg/mL Hoechst dye in assay buffer (2 M NaCl, 50 mM NaH₂PO₄, pH 7.4) were added to 50 µL of samples or calf thymus DNA standards of known concentration. Samples were incubated for 5 min at 37 °C in the dark while slowly shaking, and fluorescence was measured at 355/460 nm using POLARStar Omega microplate reader (BMG LABTECH, Ortenberg, Germany). DNA concentration of the samples was determined using a standard curve constructed with DNA solutions of known concentrations.

**Alkaline Phosphatase Activity Assay**

To determine alkaline phosphatase (ALP) activity, the culture medium was aspirated, then the cells were washed with PBS and stored at – 80 °C prior analysis. The cells were thawed and extracted by adding the cell lysis solution containing 0.5% Triton X-100 in 0.5 M 2-amino-2-methyl-1-propanol buffer with 2 mM MgCl₂ with a pH 10.3. The lysed cells were centrifuged for 5 min at 300 × g, and the ALP activity of the supernatant was determined by incubation with 0.02 M para-Nitrophenylphosphate (pNPP) substrate solution at 37 °C. The reaction time until development of yellow color was noted and the reaction was stopped by adding 0.2 M NaOH stop solution. The sample absorbance at 405 nm was measured with the POLARStar Omega microplate reader. ALP activity was determined using a standard curve constructed with p-nitrophenol solutions of known concentrations.

**Glycosaminoglycans Content Quantification**

For glycosaminoglycans (GAG) content quantification, culture medium was aspirated from BMSC cultures, samples were washed with PBS and stored at – 20 °C prior to analysis. Upon thawing, samples were digested as described for the DNA content determination in Trilineage differentiation of BMSCs on the ECM layer. Afterwards, digested samples were collected, centrifuged for 5 min at 300 × g, and GAG content of the supernatant was determined using Blyscan sulfated glycosaminoglycan assay (BioColor, B1000, Mid and East Antrim, UK) following the manufacturer’s instructions. Optical density of the samples was determined at 656 nm using the POLARStar Omega microplate reader, and GAG concentration of the samples was determined using a standard curve constructed with GAG solutions of known concentrations.

**Gene Expression Analyses**

RNA extraction was performed using the QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany), followed by DNase treatment (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Approx. 200 ng of extracted RNA was transcribed into cDNA with the GoScript™ Reverse Transcription System 100 (Promega, Madison, WI, USA) using random hexamer primers. Real-time PCR was
and then the number of adipocytes was determined. Overlapping images were taken for each of the four groups washed with distilled water. For quantification, eight non-Red O working solution was removed and samples were prepared by dissolving in 2-propanol. The samples were extensively rinsed with distilled water, and depositions 42 days after the osteogenic differentiation of BMSCs. The samples were washed with distilled water and fixed with 4% formaldehyde in PBS, placed on glass slides and kept at 37 °C overnight for optimal attachment of the sections to the glass slides.

To determine the presence of glycosaminoglycans, Alcian blue staining was performed. The samples were incubated in the Alcian blue staining solution (0.1 % in 3 % acetic acid at pH 2.5) for 30 min, rinsed with distilled water and the nuclei were counter-stained with Haemalaun (Haemalaun after Mayer, T865.3, Carl Roth, Karlsruhe, Germany). 4 µm thin sections were prepared using a microtome (Thermo Scientific Microm HM 355 S, Thermo Fisher Scientific, Waltham, MA, USA), placed on glass slides and analyzed.

To determine the presence of collagen type II, immunohistochemical staining was performed. The sections were rehydrated in a descending EtOH series and Aqua dest. Antigen retrieval was conducted using pepsin treatment (Pepsin Reagent Antigen Retriever, R2283, Merck KGaA, Darmstadt, Germany) for 10 min at 37 °C in a humidified chamber. The sections were then incubated with primary antibody against collagen type II (MS 306-P1; Collagen II Ab-3 (Clone 6B3) mouse monoclonal antibody, Thermo Fisher Scientific, Waltham, MA, USA) for one hour at RT. Upon washing, the samples were incubated with HRP-anti-mouse secondary antibody (Bright Vision poly HRP-Anti-Mouse IgG, VWRKDPVM110HRP, Immunologic, Duiven, Netherlands) for 30 min at RT. Signal was detected using NovaRed® HRP peroxidase substrate kit (ImmPTTMNova RedTM, SK4805, Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions, and the nuclei were stained with Haemalaun.

The stained samples were imaged using light microscopy (Axioplan2 imaging, Carl Zeiss Microscopy, Oberkochen, Germany and Olympus BX61VS, Olympus Corporation, Tokyo, Japan).

**Determination of Interleukin 6 Content**

Supernatants were harvested from osteogenic, adipogenic and chondrogenic BMSC cultures and their respective counterparts in control medium on the ECM and tissue culture plastic at day 3 and day 21, both times 2 days post medium change. The concentrations of interleukin 6 (IL-6) were determined by using Quantikine® ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions, using a standard curve constructed with standards of known concentrations contained in the kit.
**Results**

**hiPSC-MP-ECM Enhances Human BMSC Proliferation in Different Culture Conditions**

Human BMSCs were grown on ECM layer and on tissue culture plastic in control, osteogenic, adipogenic and chondrogenic differentiation media for up to 21 days (Fig. 1). After 5 days of culture, enhanced cell growth on the ECM layer could be observed in control, osteogenic and adipogenic media compared to cells growing on tissue culture plastic (Fig. 1A,B). At the same timepoint, BMSCs growing in micromass cultures in control medium on the ECM layer exhibited a flat and more aligned morphology compared to micromass cultures growing in control medium on tissue culture plastic. Micromass cultures in chondrogenic medium on the ECM layer remained attached and exhibited a round cell morphology, whereas micromass cultures in chondrogenic medium on tissue culture plastic started to detach from the surface and formed pellets (Fig. 1C). DNA content quantification indicated a significantly higher BMSC proliferation in control, osteogenic and adipogenic media on the ECM layer compared to tissue culture plastic after 14 and 21 days (Fig. 1D,E). For BMSC micromass cultures in control medium, no significant difference in DNA content could be observed between cultures on the ECM layer and tissue culture plastic (Fig. 1F). However, DNA content of BMSC micromass cultures in chondrogenic medium on the ECM layer was significantly higher compared to tissue culture plastic after 14 and 21 days (Fig. 1F). Taken together, our data suggest that culture on hiPSC-MP-ECM layer enhances proliferation of human BMSCs growing both in control medium in monolayer culture, as well as in differentiation media in monolayer or micromass cultures.

**hiPSC-MP-ECM Enhances Osteogenic Differentiation of Human BMSCs**

We next evaluated osteogenic differentiation markers of human BMSCs growing on ECM layer and on tissue culture plastic in control and osteogenic differentiation media for up to 42 days (Fig. 2). Gene expression analysis showed a significant upregulation of ALP, SSP1, BSP and OCN, as well as transcription factors OSX and RUNX2, after 21 days of culture in osteogenic differentiation medium on the ECM as compared to tissue culture plastic (Fig. 2A). There were no significant differences in OSX expression between the ECM and plastic groups in other differentiation conditions, whereas RUNX2 was significantly downregulated in adipogenic cultures on ECM as compared to tissue culture plastic (Supplementary Fig. 1). Analysis of ALP enzyme activity after 14 and 21 days of culture showed a significant upregulation in osteogenic differentiation medium on the ECM as compared to tissue culture plastic (Fig. 2B), whereas ALP activity normalized to DNA content was not significantly different between the two groups (Supplementary Fig. 2). Furthermore, Alizarin red staining showed a markedly higher calcium deposition by human BMSCs cultured in osteogenic differentiation medium for 42 days on the ECM as compared to the tissue culture plastic (Fig. 2C). Taken together, these data show a strong enhancement of early and late osteogenic markers in BMSCs differentiating on the ECM layer.

**hiPSC-MP-ECM Reduces Adipocyte Formation by Human BMSCs**

We next determined adipogenic differentiation markers of human BMSCs growing on ECM layer and on tissue culture plastic in control and adipogenic differentiation media for up to 21 days (Fig. 3). Expression of all four adipogenic marker genes was markedly higher in BMSCs growing in adipogenic differentiation medium compared to control medium on both ECM layer and tissue culture plastic after 21 days of culture. In adipogenic differentiation medium, gene expression levels of PPARG and ADIPOQ genes were comparable between BMSCs cultured on the ECM layer and tissue culture plastic, whereas FABP 4 and LPL genes indicated a trend of lower gene expression on the ECM layer compared to tissue culture plastic (Fig. 3A).

Interestingly, quantification of cells stained positively by Oil Red O showed a significant decrease in the formation of lipid-containing adipocytes after 10, 15 and 21 days of culture in adipogenic medium on the ECM layer compared to tissue culture plastic (Fig. 3B,C). No lipid-containing adipocytes were found in control medium on both culture surfaces. Taken together, these data suggest a decrease in adipogenic differentiation of BMSCs on the ECM layer.

**hiPSC-MP-ECM Enhances Chondrogenic Differentiation of Human BMSCs**

We next determined chondrogenic differentiation markers of human BMSCs growing on ECM layer and on tissue culture plastic in micromass cultures in control and chondrogenic differentiation media for up to 21 days (Fig. 4). Gene expression analysis showed a significant upregulation of Col II, Sox 9 and ACAN gene expression after 21 days in chondrogenic culture conditions on the ECM as compared to tissue culture plastic (Fig. 4A). In contrast, gene expression of Col X showed no significant dif-
Fig. 1. Enhanced proliferation of human BMSCs on ECM layer in different culture conditions. (A–C) Representative images of enhanced cell growth after 5 days of culture on the ECM layer compared to standard tissue culture plastic in control, osteogenic, adipogenic and chondrogenic media. Scale bars: 200 µm. Group labels: Ctrl–control medium, Osteo–osteogenic differentiation medium, Adipo–adipogenic differentiation medium and Chondro–chondrogenic differentiation medium. (D–F) DNA content quantification of BMSC cultures on the ECM layer and tissue culture plastic during osteogenic (D), adipogenic (E) and chondrogenic (F) differentiation. Group labels: PL ctrl–plastic with control medium, ECM ctrl–ECM with control medium, PL osteo–plastic with osteogenic differentiation medium, ECM osteo–ECM with osteogenic differentiation medium, PL adipo–plastic with adipogenic differentiation medium, ECM adipo–ECM with adipogenic differentiation medium, PL chondro–plastic with chondrogenic differentiation medium, ECM chondro–ECM with chondrogenic differentiation medium. Data represents mean ± SD (n = 4–6). Statistically-significant differences between the groups were evaluated using two-way ANOVA, followed by Tukey’s multiple comparison test, and are marked with: *p < 0.05; ***p < 0.001; ****p < 0.0001. BMSCs, bone marrow-derived MSCs; ECM, extracellular matrix.
Fig. 2. Enhanced osteogenic differentiation of human BMSCs on ECM layer. (A) Relative gene expression levels of osteogenic markers alkaline phosphatase (ALP), osteopontin (SSP1), bone sialoprotein (BSP) osteocalcin (OCN), osterix (OSX) and runt related transcription factor 2 (RUNX2) after 21 days of culture. (B, C) ALP activity after 14 and 21 days of culture. (A, B, C) Group labels: PL ctrl–plastic with control medium, ECM ctrl–ECM with control medium, PL osteo–plastic with osteogenic differentiation medium, ECM osteo–ECM with osteogenic differentiation medium. Data represents mean ± SD (n = 4–6). Statistically-significant differences between the groups were evaluated using Kruskal–Wallis test, followed by Dunn’s multiple comparison test (Panel A), or two-way ANOVA, followed by Tukey’s multiple comparison test (Panel B), and are marked with: *p < 0.05; **p < 0.01; ****p < 0.0001. (C) Alizarin Red staining for mineral deposition (red deposits) after 42 days of culture. Representative images are shown (n = 3). Scale bars: 200 µm.

difference between the micromass cultures. Moreover, after 21 days of culture, we found a significant increase in GAG deposition by the BMSCs in chondrogenic differentiation medium on the ECM compared to tissue culture plastic (Fig. 4B). GAG deposition normalized to DNA content was not significantly different between the two groups (Supplementary Fig. 2). Histological staining of one randomly sampled micromass tissue per group was conducted to evaluate the matrix deposition pattern (Fig. 4C, D). The increase in GAG content corresponded with the Alcian blue histological staining, which showed a more uniform GAG deposition when human BMSCs were cultured on the ECM as compared to tissue culture plastic under chondrogenic induction (Fig. 4C). Furthermore, a more uniform collagen type II deposition could be observed in the micromass sample cultured for 21 days in differentiation medium on the ECM (Fig. 4D). Taken together, these data show an enhancement of chondrogenic differentiation of BMSCs on the ECM layer with no concomitant increase in hypertrophy marker expression.
Fig. 3. Reduced adipocyte formation by human BMSCs on ECM layer. (A) Relative gene expression levels of adipogenic markers peroxisome proliferator activated receptor gamma (PPARG), adiponectin (ADIPOQ), fatty acid binding protein 4 (FABP4) and lipoprotein lipase (LPL) after 21 days of culture. (B) Adipocyte formation during 21 days of culture. (A,B) Group labels: PL ctrl–plastic with control medium, ECM ctrl–ECM with control medium, PL adipo–plastic with adipogenic differentiation medium, ECM adipo–ECM with adipogenic differentiation medium. Data represents mean ± SD (n = 4 or 8). Statistically-significant differences between the groups were evaluated using Kruskal–Wallis test, followed by Dunn’s multiple comparison test (Panel A), or two-way ANOVA, followed by Tukey’s multiple comparison test (Panel B), and are marked with: ****p < 0.0001. (C) Oil red O staining for lipid-laden adipocyte formation (red) after 21 days of culture. Representative images are shown (n = 3). Scale bars: 200 µm.

Cultivation on hiPSC-MP-ECM Modulates IL-6 Secretion by Differentiating Human BMSCs

To determine whether exposure to the hiPSC-MP ECM layer affects BMSC secretory activity, we evaluated secretion of IL-6 in supernatants of human BMSCs after 3 and 21 days of differentiation on the ECM layer and on tissue culture plastic (Fig. 5). After 3 days of culture, IL-6 concentration was the highest in monolayer and micromass cultures growing in control medium, with no significant differences between the ECM layer and tissue culture plastic (Fig. 5). BMSCs in osteogenic and adipogenic differentiation media in monolayer cultures showed comparable low IL-6 concentrations on ECM layer and tissue culture plastic. In contrast, BMSC micromass cultures in chondrogenic differentiation medium showed a significant increase in IL-6 concentration on the ECM layer compared to tissue culture plastic. After 21 days of culture, IL-6 concentration of monolayer culture groups in control medium was lower compared to the earlier timepoint with no significant difference between the ECM layer and tissue culture plastic. In contrast, BMSC micromass cultures in control medium exhibited a significantly higher IL-6 concentration on tissue culture plastic as compared to ECM layer after 21 days. IL-6 concentration was also significantly higher in monolayer cultures in osteogenic differentiation medium on tissue culture plastic compared to culture on ECM layer, whereas concentrations of IL-6 in adipogenic and chondrogenic differentiation media were low on both culture surfaces. Taken together, our data indicate that cultivation on the ECM layer modulates the release of IL-6 compared with tissue culture plastic, and the cell secretory responses depend on culture conditions and duration of culture.

Discussion

Engineered ECMs are biological scaffolds that mimic the complex structure and signaling of native tissues and are therefore intensely investigated for bone and osteochondral tissue engineering and regeneration (Benders et al., 2013; Förster et al., 2012; Zhang et al., 2016). ECM engineering utilizing primary MSCs faces challenges due to donor variability, limited cell numbers, limited cell expansion potential and loss of regenerative capacity after in vitro expansion. In contrast, hiPSCs represent a scalable source for production of youthful human progenitor cells such as hiPSC-MPs and bioactive tissue components, thus supporting eventual clinical translation. In our previous study, we engineered ECM from hiPSC-MPs and found that it strongly enhanced the osteogenic capacity of primary hu-
Fig. 4. Enhanced chondrogenic differentiation of human BMSCs on ECM layer. (A) Relative gene expression levels of chondrogenic markers collagen type II (COL II), SRY-box 9 (Sox9), collagen type X (COL X) and aggrecan (ACAN) after 21 days of culture. (B) GAG deposition after 21 days of culture. (A,B) Group labels: PL ctrl–plastic with control medium, ECM ctrl–ECM with control medium, PL chondro–plastic with chondrogenic differentiation medium, ECM chondro–ECM with chondrogenic differentiation medium. Data represents mean ± SD (n=4). Statistically-significant differences between the groups were evaluated using Kruskal–Wallis test, followed by Dunn’s multiple comparison test (Panel A), or two-way ANOVA, followed by Tukey’s multiple comparison test (Panel B), and are marked with: ***p < 0.001; ****p < 0.0001. (C) Alcian blue staining for GAG deposition (blue) after 21 days of culture. (D) Immunohistochemical staining for collagen type II deposition (brown) after 21 days of culture. (C,D) Staining of one randomly sampled micromass tissue per group is shown. Scale bars: 100 µm.

Fig. 5. Effects of culture on ECM layer on IL-6 secretion during BMSC differentiation. Concentrations of IL-6 after 3 and 21 days of culture were determined by ELISA. Group labels monolayer cultures: Ctrl–plastic or ECM with control medium, Osteo–plastic or ECM with osteogenic differentiation medium, Adipo–plastic or ECM with adipogenic differentiation medium. Group labels micromass cultures: Ctrl–plastic or ECM with control medium, Chondro–plastic or ECM with chondrogenic differentiation medium. Statistically significant differences between the groups were evaluated using two-way ANOVA, followed by Tukey’s multiple comparison test, and the differences between ECM and plastic groups are marked with: *p < 0.05; ****p < 0.0001.
human BMSCs from young adult- and aged donors in two- and three-dimensional culture models (Hanetseder et al., 2023). Therefore, in the current study, we investigated (using cells from the same young adult donor) whether culture on hiPSC-MP-ECM affects the BMSC trilineage differentiation and associated secretory activity compared to BMSCs cultured on standard tissue culture plastic. We found that the hiPSC-MP-ECM strongly enhanced cell growth in control and differentiation culture conditions. Furthermore, BMSC culture on the ECM enhanced osteogenic and chondrogenic differentiation, while reducing adipocyte formation. BMSC culture on hiPSC-MP-ECM layer also significantly affected the secretion of IL-6 after 3 and 21 days of differentiation, pointing to the importance of the ECM microenvironment for BMSC trophic activity.

The ability of MSC-derived ECMs to significantly enhance cell expansion in growth media compared to standard tissue culture plastic has already been shown in several studies (Chen et al., 2007; He and Pei, 2013; Lai et al., 2010; Ng et al., 2014; Yang et al., 2018). In line with these studies and our prior work (Hanetseder et al., 2023), we found that hiPSC-MP-ECM enhanced BMSC proliferation in monolayer cultures also in control medium. Furthermore, enhanced BMSCs proliferation was noted in all three differentiation conditions, i.e., in osteogenic and adipogenic differentiation media in monolayer cultures and in chondrogenic differentiation medium in micromass cultures. To our knowledge, our study is the first to find enhanced cell proliferation during osteogenic-, chondrogenic- and adipogenic differentiation on the same cell-derived ECM layer compared to standard tissue culture plastic. Ng et al. also showed that late-passage adult MSC properties were significantly improved on fetal MSCs-derived ECM, including cell proliferation, stemness and osteogenic, adipogenic and chondrogenic differentiation compared to tissue culture plastic. Together, these studies and our work suggest that ECMs from biologically-young cells could be a promising substrate to improve *ex vivo* expansion of autologous MSCs for clinical applications (Chen et al., 2007; Ng et al., 2014). Furthermore, our current study indicates that differentiation of MSCs on the ECM presents an additional advantage, as it further increases the yield of differentiating cells.

ECM in the current study was generated using the same procedure as in our previous study, which confirmed the maintenance of collagen type I, collagen type IV, laminin and fibronectin in the ECM after the decellularization procedure (Hanetseder et al., 2023). It was previously shown that individual ECM proteins such as collagens, fibronectin and laminin have a proliferative effect on BMSCs (Becerra-Bayona et al., 2012; Kihara et al., 2006; Linsley et al., 2013; Mizuno et al., 2000; Nieto-Nicolau et al., 2020; Salaszyk et al., 2004). Furthermore, we and others found that the cultivation on engineered ECM resulted in stronger enhancement of adult BMSC growth compared to single protein substrates (Hanetseder et al., 2023; Ng et al., 2014). It remains to be determined whether this effect is mainly due to the synergistic signaling activity of individual ECM components, or whether this is also affected by the mechanical properties of the ECM mimicking the native tissue (Marinkovic et al., 2020). Interestingly, prior studies showed that despite unique matrisome signatures of individual ECMs derived from BMSCs, adipose derived MSC or neonatal dermal fibroblasts, the overall stimulatory effects on MSC proliferation were comparable (Prewitz et al., 2013; Ragelle et al., 2017).

Engineered ECMs were previously found to enhance osteogenic, chondrogenic and adipogenic differentiation in various study setups (Ang et al., 2014; Carvalho et al., 2019; Cheng et al., 2009; Jeon et al., 2018; Lu et al., 2011). With regards to osteogenic differentiation, ECM layers engineered from young adult human BMSCs enhanced alkaline phosphatase activity, gene expression of *Col1A1, RUNX 2* and *ALP* and mineralization of human BMSCs (Carvalho et al., 2019). We have already shown in our previous study that both hiPSC-MP-ECM layer and silk scaffold coating increased osteogenic gene expression, bone matrix proteins deposition and mineralization of BMSCs from young adult and especially from aged donors (Hanetseder et al., 2023). In line with these findings, enhancement of young adult BMSC osteogenic differentiation on the ECM layer was also observed in the current study, using the same donor cells in an independent experiment. We noted significantly increased ALP activity after 14 and 21 days, that could be attributed to increased cell proliferation, as well as significantly increased osteogenic gene expression after 21 days and matrix mineralization after 42 days. While some differences in ALP activity responses of young adult BMSCs derived from the same donor were noted between our two studies, the overall ALP activity levels reached were similar between the studies. These differences might be attributed to inter-experimental variation, as it is known that increase in ALP activity is an early marker of osteogenic differentiation, which changes dynamically with the progress of differentiation (Malaval et al., 1994). Significant increase in expression of transcription factors RUNX2 and OSX in osteogenic conditions, further induced by the ECM, is in line with their roles in osteogenic lineage induction and osteoblast differentiation (Chan et al., 2021). Interestingly, RUNX2 expression was significantly decreased in adipogenic cultures on the ECM, suggesting the ECM acts on adipogenic/osteogenic differentiation regulatory network (Rauch et al., 2019).

Furthermore, ECM layers derived from osteogenic- or adipogenic-induced MSCs were shown to specifically enhance the corresponding lineage in re-seeded MSCs through integrated structural and regulatory proteins (Ang et al., 2014; Carvalho et al., 2019; Jeon et al., 2018). Ang et al. showed that collagen type IV is one of the major components of basement membranes, which provides...
structural support for mature lipid-laden adipocytes (Ang et al., 2014). They also indicated that under macromolecular crowding conditions, adipogenic-induced human BMSCs shifted from collagen type I toward collagen type IV production and reduced fibronectin deposition in their ECM. In our previous study, we found adipocyte formation in osteogenic cultures of aged donor BMSCs on tissue culture plastic. However, no adipocytes were noted on hiPSC-MP-derived ECM (Hanetseder et al., 2023). We were therefore interested in how the ECM would affect BMSC induction into adipogenic lineage. Interestingly, we found a comparable expression of genes for adipogenic master regulator PPARγ and adiponectin (ADIPOQ), a hormone which is thought to be active further downstream in the adipokine signaling pathway (Menssen et al., 2011) in BMSCs cultured on the ECM as compared to tissue culture plastic. Genes coding for fatty acid-binding protein 4, implicated in early adipogenesis, and lipoprotein lipase (LPE) (Menssen et al., 2011) exhibited a trend of lower expression in BMSCs cultured on the ECM layer compared to tissue culture plastic. However, a significant reduction in the formation of mature, lipid-laden adipocytes was found on the ECM layer compared to tissue culture plastic in adipogenic differentiation medium. It was shown previously that osteogenesis and adipogenesis are inversely related processes, where osteogenesis depends on commissioning of a network of MSC transcription factors that act as repressors of adipogenesis (Rauch et al., 2019; Suo et al., 2022). In line with this, our data suggest that the hiPSC-MP-ECM signals a shift in adult BMSC differentiation balance from adipogenic to osteogenic differentiation.

In prior studies, the stimulatory effect of cell-generated ECM on chondrogenic differentiation was demonstrated for hybrid ECM scaffolds and scaffolds composed fully of ECM (Cheng et al., 2009; Jin et al., 2007; Lu et al., 2011; Tang et al., 2013, 2014; Yuan et al., 2013). These scaffolds were shown to contain ECM components that activate integrin signaling as well as pro-chondrogenic growth factors that are retained in the matrix after chemotherapies that activate integrin signaling as well as pro-chondrogenic transcription factors that act as repressors of adipogenesis (Rauch et al., 2019; Suo et al., 2022). In line with this, our data suggest that the hiPSC-MP-ECM signals a shift in adult BMSC differentiation balance from adipogenic to osteogenic differentiation.

The results presented here are the first step in evaluating the potential of hiPSC-MP-derived ECM to modulate in micromass culture has not been previously assessed. We found increased gene expression of chondrogenic markers, including the master regulator SOX9, as well as collagen type II and aggrecan, both markers of the mature chondrocyte phenotype (Mwale et al., 2006) in BMSCs cultured on the ECM compared to tissue culture plastic. In contrast, no increase was found in collagen type X gene expression, suggesting that the ECM does not facilitate BMSC differentiation towards hypertrophy compared to tissue culture plastic (Mueller and Tuan, 2008; Yang et al., 2019). Enhanced expression of cartilaginous specific gene markers in our BMSC micromass cultures on the ECM layer also correlated with increased and more uniform collagen type II and GAG deposition, as found in previous studies in pellet-micromass culture model (Lu et al., 2011; Yang et al., 2018). However, the full impact of ECM on chondrogenic induction of BMSCs and the potential for stable cartilage formation versus induction of endochondral ossification pathway needs to be further evaluated.

Finally, we aimed to assess how BMSC secretory activity is affected by differentiation on the ECM layer. We focused on evaluating the secretion of IL-6, a cytokine with pleiotropic roles in the regulation of the immune system (Dorronsoro et al., 2020). In the context of osteogenic differentiation, it was previously reported that IL-6 secretion is high in undifferentiated BMSCs, whereas it decreases during BMSC differentiation (Pricola et al., 2009). It was also suggested that IL-6 inhibited BMSC differentiation (Bian et al., 2010; Pricola et al., 2009). In the context of cartilage repair, increased levels of IL-6 were shown to promote the anabolic metabolism of chondrocytes, and to be protective for neocartilage formation (Tsudhida et al., 2012). In our study, IL-6 secretion was highest in BMSCs cultured in control medium in monolayer and micromass cultures. In contrast to a prior report (Pricola et al., 2009), we found a significant increase in IL-6 release by BMSCs in the later stage of osteogenic differentiation when cells were cultured on standard tissue culture plastic compared to the ECM layer. It was previously shown that growth plate- but not articular cartilage-derived ECM induced production of IL-6 and TNF, both of which have a pro-osteogenic role (Glass et al., 2011; Huang et al., 2018; Kurozumi et al., 2019). In chondrogenic micromass cultures, IL-6 secretion was also significantly enhanced by the ECM layer at an earlier point of induction, coinciding with enhanced cell proliferation. At the later stage of chondrogenic differentiation, IL-6 secretion was practically undetectable on both culture surfaces. Together, our data suggest that the microenvironment provided by the ECM layer as well as the differentiation medium signals affect the BMSC secretory activity. Modulation of BMSC secretory activity thus needs to be considered within the context of BMSC-based regenerative therapies utilizing engineered ECM.
trilineage differentiation of BMSCs for potential applications in bone and osteochondral regenerative therapies. In future studies, the key ECM components responsible for enhancing BMSC proliferation and modulating trilineage differentiation, their stability, and the molecular mechanisms will need to be elucidated. Also, it remains to be determined whether BMSC donor age affects the cells’ sensitivity to the signals provided by hiPSC-MP-ECM, since we only tested the effects of hiPSC-MP-ECM on BMSCs derived from a young adult donor, which exhibited a strong differentiation potential. Decline in osteogenic differentiation capability has been found in BMSCs from aged donors, and it would be interesting to evaluate whether the shift in differentiation potential found in the young donor BMSCs could also be achieved for chronologically aged BMSCs.

Conclusions

In conclusion, we have shown that osteogenic and chondrogenic differentiation of young adult human BMSCs can be strongly enhanced by culturing them on an ECM layer engineered \textit{in vitro} from hiPSC-MPs. We have also shown that hiPSC-MP ECM enhanced cell growth in different culture conditions while reducing lipid-laden adipocyte formation. However, this study has not undertaken a quantitative analysis of the ECM components and their role in stimulatory actions. It remains to be established which particular components, along with the structure and mechanical properties of the ECM, are contributing to the stimulatory effects on BMSC growth and differentiation. With hiPSCs representing a potentially unlimited and scalable human cell source, developing tissue engineering strategies employing hiPSC-engineered ECM could be a feasible and attractive approach for osteochondral defect repair.

List of Abbreviations

BMSCs, bone marrow-derived MSCs; ECM, extracellular matrix; MSCs, mesenchymal stromal cells; 3D, three-dimensional; hiPSC-MPs, human MSC-like progenitors; KO-DMEM, KnockOut Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; P/S, penicillin-streptomycin; b-FGF, basic fibroblast growth factor; DMEM-HG, Dulbecco’s modified Eagle’s medium high glucose; A2P, ascorbic acid 2-phosphate; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β; ALP, alkaline phosphatase; pNPP, para-Nitrophenylphosphate; GAG, glycosaminoglycans; PPARG, peroxisome proliferator activated receptor gamma; ADIPOQ, adiponectin; FABP4, fatty acid binding protein 4; LPL, lipoprotein lipase; COL2A1, Collagen type II; ACAN, Aggrecan; COL10A1, Collagen type X; Sox9, SRY-box 9; RUNX2, runt related transcription factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-6, interleukin 6; SSP1, osteopontin; BSP, bone sialoprotein; OCN, osteocalcin; OSX, osterix.

Availability of Data and Materials

All datasets and materials gathered are incuded in the published manuscript.

Author Contributions

Conceptualization and study design, DH, HR and DMP; Investigation, methodology and visualization, DH and BS; Formal analysis, DH, HR and DMP; Writing—original draft, DH and DMP; Writing—review & editing, DH, HR, BS and DMP; Project administration, DMP; Project supervision, HR and DMP. All authors were informed about each step of manuscript processing and have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgments

We would like to thank our colleagues from Ludwig Boltzmann Institute for Traumatology for their suggestions and support of the project, especially Vanessa Goechsl for her help with cell culture. We would like to thank Regina Brunauer and Simon Sperger for their suggestions with manuscript writing. We further thank the New York Stem Cell Foundation Research Institute for providing the human induced pluripotent stem cell line 1013A and its mesenchymal progenitors. The graphical abstract was created with the online tool BioRender.com.

Funding

This work has received funding from the European Union’s Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie actions (grant agreement No. 657716) and Transforming European Industry call H2020-NMBP-TR-IND-2020 (grant agreement No. 953134), as well as by the FFG Industrienahe Dissertation program (grant agreement No. 867803) and the FFG Production of the Future program (grant agreement No. 877452).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.22203/eCM.v047a08.

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Editor’s note: The Scientific Editor responsible for this paper was Fergal O’ Brien.