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ORIGINAL ARTICLE

Response of Primary Human Bone Marrow Mesenchymal Stromal Cells and Dermal Keratinocytes to Thermal Printer Materials In Vitro

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Abstract Advancement in thermal three-dimensional printing techniques has greatly increased the possible applications of various materials in medical applications and tissue engineering. Yet, potential toxic effects on primary human cells have been rarely investigated. Therefore, we compared four materials commonly used in thermal printing for bioengineering, namely thermally printed acrylonitrile butadiene styrene, MED610, polycarbonate, and polylactic acid, and investigated their effects on primary human adult skin epidermal keratinocytes and bone marrow mesenchymal stromal cells (BM-MSCs) in vitro. We investigated indirect effects on both cell types caused by potential liberation of soluble substances from the materials, and also analyzed BM-MSCs in direct contact with the materials. We found that even in culture without direct contact with the materials, the culture with MED610 (and to a lesser extent acrylonitrile butadiene styrene) significantly affected keratinocytes, reducing cell numbers and proliferation marker Ki67 expression, and increasing glucose consumption, lactate secretion, and expression of differentiation-associated genes. BM-MSCs had decreased metabolic activity, and exhibited increased cell death in direct culture on the materials. MED610 and acrylonitrile butadiene styrene induced the strongest expression of genes associated to differentiation and estrogen receptor activation. In conclusion, we found strong cell-type-specific effects of the materials, suggesting that materials for applications in regenerative medicine should be carefully selected not only based on their mechanical properties but also based on their cell-type-specific biological effects.

Keywords Biocompatibility · Keratinocyte · Bone marrow mesenchymal stromal cell · Thermal printer material · MED610

1 Introduction

Various inorganic and organic structural materials have been used in conventional cell culture and tissue engineering for regenerative medicine purposes or as traditional implants [1]. The biocompatibility of some materials has been investigated; however, most studies used immortalized cell lines [2, 3] and not primary cells, and did not compare various materials with each other. Yet, particularly with regard to clinically targeted tissue engineering applications and pharmaceutical drug testing models, potential negative effects of these materials on primary human cells should be determined and avoided. Therefore, we chose to investigate materials that are used frequently in thermal printing for tissue engineering or in the construction of medical devices, namely acrylonitrile butadiene styrene (ABS), polycarbonate (PC), polylactic acid (PLA),

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and MED610 (MED). We studied in detail the effects of these materials on primary human adult cells in order to have a more accurate model system compared to cell lines. We intentionally used cell types of epidermal and mesodermal origin, i.e., adult skin epidermal keratinocytes and bone marrow mesenchymal stromal cells (BM-MSCs), in order to detect potential cell-type-specific effects. The four materials have been used in cell culture for various applications based on their specific properties. Three-dimensional (3D) scaffolds for tissue engineering are frequently created from PLA due to its structural strength, biodegradability, bio-absorbability, and ability to form hybrid composites [4, 5]. ABS is a thermoplastic that has been used to create molds for applications such as an ear-shaped human skin cell culture [6] and the creation of tympanic membranes of bovine chondrocytes [7]. A recent study comparing 3D-printed scaffolds of PLA and ABS for the regeneration of cartilage and nucleus pulposus [8] did not find significant differences between these two materials in terms of cell viability, protein production, and tissue ingrowth. PC [3] and MED are materials used in orthodontics. MED is a proprietary, medically approved clear thermoplastic recommended for use in medical devices such as hearing aids, as well as dental and orthodontic models. Cell culture or animal studies using this material have not been published. According to the manufacturer, this material has been tested for biocompatibility and has medical approval.

Although many materials have been used as two-dimensional (2D) surfaces or 3D scaffolds in cell culture and tissue engineering, these have not been compared in terms of their biocompatibility or toxicity. Also, a major drawback in toxicity testing of materials is that most studies use immortalized cell lines; human primary cells have been used rarely to investigate the toxic effects of materials. For example, a study by Laluppa et al. investigated the response of primary hematopoietic progenitors to various materials, including PC, glass, metals, and cellulose acetate [9]. Their results demonstrated that many materials approved for hematopoietic cell contact were not suitable for cell culture.

In this study, we investigated the effects of four materials on the cell viability, number, metabolism, and gene expression of two primary human adult cell types. For both cell types, we determined indirect effects caused by potential liberation of soluble substances from the materials, and also measured effects on BM-MSCs in direct contact with the materials. Keratinocytes could not be cultured directly on materials, as they require a collagen coating to enable attachment. We found that the culture with MED, and to a lesser extent ABS, significantly affected keratinocytes, while PLA had minor effects and PC did not elicit any effects. MED significantly reduced keratinocyte numbers, increased glucose consumption and lactate secretion, and suppressed proliferation- and induced differentiation-associated genes of both cell types. BM-MSCs were less responsive to the materials in indirect culture, but culture on the materials caused significant effects as well. The strongest effects were observed for MED and ABS. Our results indicate that PC and PLA have better biocompatibility than that of MED and ABS, which significantly affected human primary cells. These strong biological effects of specific materials should be considered when applying the materials for tissue engineering.

2 Materials and Methods

2.1 Cell Isolation and Culture

Human adult BM-MSCs were purchased frozen (Lonza, Walkersville, MD, and StemCell Technologies, Vancouver, Canada). Cells were cultured initially for two to three passages in standard tissue-culture-treated polystyrene cell culture flasks (Corning Life Sciences, Lowell, MA) in Mesencult medium (StemCell Technologies), including supplements supplied by the manufacturer, 5 % human serum (Lonza), and an antibiotic–antimycotic mix (Life Technologies, Carlsbad, CA). For testing of materials, cells were cultured in phenol-red-free DMEM, including 10 % fetal bovine serum (FBS), an antibiotic–antimycotic mix, and glutamax (all Life Technologies).

Human adult keratinocytes were isolated from abdominal skin biopsies derived from tissue donations (Department of Surgery, University of Pittsburgh Medical Center, PA) with informed consent of the donors and approval of the University of Pittsburgh Institutional Review Board. Skin biopsies (thickness: 0.2 mm) were obtained using a manual dermatome (Teleflex, Limerick, PA). The skin was cut into squares of approximately 6 mm × 6 mm using forceps and a scalpel. Pieces of skin were incubated at 37 °C with dispase-II-solution (Life Technologies) for about 40–50 min. The epidermis was separated from the dermis using two 16-G needles and digested at 37 °C with 0.05/0.02 % Trypsin/EDTA-solution (Life Technologies). The digestion was stopped by adding 5 % FBS (PAA Laboratories, Dartmouth, MA, USA) into the tube. Single-cell epidermal suspensions were obtained after filtering through a 40-μm cell sieve (Corning Life Sciences). Cells were centrifuged and re-suspended in Epilife medium, including the manufacturer provided EDGS Growth Supplement and an antibiotics/antimycotic mix (Life Technologies). Cell viability and numbers were determined by trypan blue (Life Technologies) exclusion in a Neubauer chamber. Cells were plated in conventional cell culture flasks (Corning Life Sciences) coated with 50 μg/cm^2 rat-

tail collagen-1 (Corning Life Sciences). For material testing, keratinocytes were used after two to three passages and cultured in phenol-red-free, supplemented Epilife medium.

2.2 Material Testing

Four materials were investigated: ABS, MED, PC (Stratasys, Eden Prairie, MN), and PLA (PrintrBot, Lincoln, CA). The materials were thermally printed (PrintrBot) in plates with a thickness of about 1 mm, and circular disks with a diameter of 11 mm were cored. The materials were briefly cleaned by rinsing with 100 % ethanol and washed five times with de-mineralized water. The materials were placed in cell culture plates and sterilized with ethylene oxide gas sterilization.

Cells were plated at a density of 10,000 cells per cm^2. Cultures were performed using 0.4-µm pore size polyester 12-well Transwell insert cell culture plates with a polystyrene housing (Corning Life Sciences). Figure 1 provides a schematic drawing of the experimental layout. Negative controls included wells containing neither cells nor materials [Fig. 1-I (a)], and positive controls included cells seeded in inserts without the materials [Fig. 1-I (b)]. Additional negative controls included wells containing the materials only (without cells) [Fig. 1-I (c)]. BM-MSCs were either seeded on inserts with materials placed at the bottom of the dish [Fig. 1-I (d)], or seeded directly on materials placed in inserts [Fig. 1-I (e)]. This setup allowed for the investigation of the effects on cells caused by direct contact with the materials and liberation of substances from the materials, as well as the effects on cells caused by potential liberation of substances from the materials only. Keratinocytes were cultured in a modified setup as they could not be tested for the effects caused by direct physical contact with the materials because previous experiments had shown that keratinocytes require a collagen-1 coating for their culture (direct culture on either inserts or materials was not successful). Negative controls included wells containing neither cells nor materials [Fig. 1-II (a)], and positive controls included cells seeded on collagen-1-coated bottoms of dishes but without materials in inserts [Fig. 1-II (b)]. Additional negative controls included wells containing only the materials in inserts (without cells) [Fig. 1-II (c)]. Keratinocytes were seeded on collagen-1-coated bottoms of cell culture dishes and the materials were placed in the inserts for the investigation of the effects on cells caused by potential liberation of substances from the materials [Fig. 1-II (d)].

Cells were cultured in incubators providing 95 % humidity and 5 % CO_2. All cells and medium samples were analyzed 48 h after plating.

2.3 Fluorescence Microscopy and Confocal Imaging

To localize and characterize cells in direct culture on the various materials, BM-MSCs were analyzed using

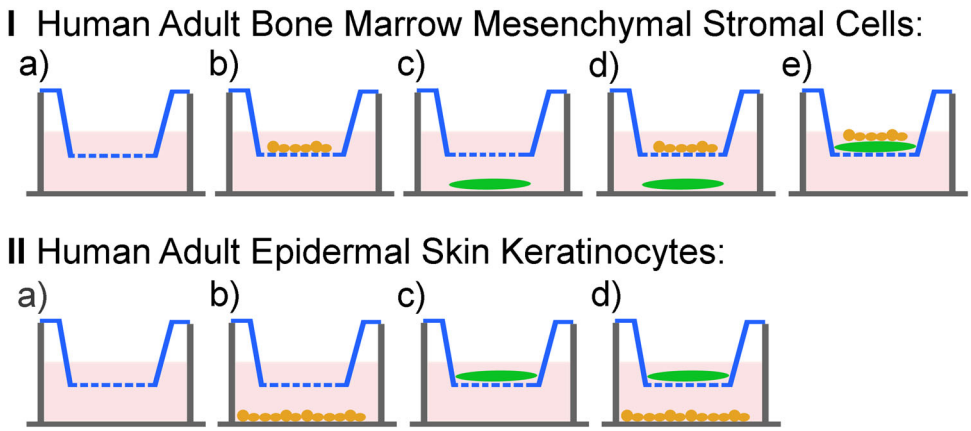

Fig. 1 Schematic layout of experimental setup. Cultures of cells (*brown*) were performed in wells (*dark grey*) of cell culture plates that contained detachable polyester inserts (*blue*) with 0.4-µm pore size. The size of the pores permits unrestricted exchange of medium (*pink*) but not cells. The setup allowed for investigation of effects on cells caused by direct contact with materials (*green*) and liberation of substances from materials, as well as for investigation of effects on cells caused by potential liberation of substances from materials only. For bone marrow mesenchymal stromal cells (I) negative controls included wells containing neither cells nor materials (**I a**), and positive controls included cells seeded in inserts without materials (**I b**). Additional negative controls included wells containing materials only (without cells) (**I c**). Cells (I) were seeded on inserts with materials placed at the bottom of the dish (**I d**) or seeded directly on materials placed in inserts (**I e**). Keratinocytes (II) were cultured on collagen-1-coated bottoms of cell culture dishes. Negative controls included wells containing neither cells nor materials (**II a**), and positive controls included cells seeded on bottoms of dishes without materials in inserts (**II b**). Additional controls included wells containing only materials in inserts (without cells) (**II c**). Keratinocytes were seeded on bottoms of cell culture dishes and materials were placed in inserts for investigation of effects on cells caused by potential liberation of substances from materials (**II d**). Proportions are not to scale

fluorescence microscopy. After 48 h in culture, materials with cells were fixed with 4 % para-formaldehyde. Washing steps between stainings included three washes with phosphate-buffered saline. Nuclei of cells were stained with the nucleic-acid-specific fluorescence dye DAPI and with cytoskeleton F-actin-specific AF568-phalloidin (Thermo Fisher Scientific, Waltham, MA). To investigate the surface topography of the various materials, samples were imaged using high-magnification bright-light microscopy. Images were acquired using confocal microscopy with an Olympus Fluoview 1000 imaging system (Center Valley, PA). Images were assembled using Adobe Photoshop CS3 Extended version 10.0.1 software (Adobe Systems, San Jose, CA).

2.4 Gene Expression Analyses

To investigate the effects of materials on differentiation, apoptosis, and necrosis of cells in indirect and direct culture, gene expression was studied with quantitative real-time polymerase chain reaction (PCR). Cells in culture were lysed directly with RLT buffer, and RNA was isolated using shredder and isolation columns of RNeasy-mini kits (Qiagen, Valencia, CA) including on-column DNase treatment. RNA was reverse transcribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Gene expression was analyzed using real-time PCR with the StepOnePlus system and software, pre-designed Taqman probe assay mixes, and gene expression PCR master mixes (Applied Biosystems). Beta-actin served as the housekeeping gene for internal normalization. For each sample, three technical repeats were performed. Primary human cells from the initial plating day (day 0) served as the relative quantitative normalizer. Expression was quantified using the ddCt method.

2.5 Glucose and Lactate Measurement

Metabolic activities of cells were monitored from medium samples collected 48 h after plating. Glucose and lactate concentrations were measured using a Cobas b221 blood gas analyzer (Roche Diagnostics, Indianapolis, IN).

2.6 Indirect Cell Viability Assay by LDH

To investigate whether the materials affected cell viability during culture, we analyzed indirect cell viability daily. Cell viability was measured indirectly by quantifying the activity of released lactate dehydrogenase (LDH) in culture medium caused by loss of cell membrane integrity. Culture media were analyzed with the QuantiChrom LDH Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. Optical density (OD) of samples was read in 96-well plates at 565 nm, using a Synergy H1 Hybrid multi-mode reader and Gen5 data analysis software (BioTek, Winooski, VT).

2.7 Numbers of Viable Cells Obtained by MTT Assay

The numbers of viable cells after 48 h of culture were determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) substrate assay (Life Technologies) according to manufacturer's instructions. Viable cells are able to form water-insoluble formazan from the substrate. 1.2 mM MTT was added to the cell culture medium, and cells were incubated for 4 h. The water-insoluble formazan was solubilized in dimethyl sulfoxide (DMSO); Sigma-Aldrich, St. Louis, MO), incubated for 10 min at room temperature, and absorbance was read at 540 nm using a Synergy H1 Hybrid multi-mode reader and Gen5 data analysis software (BioTek). Linear standard curves for BM-MSCs and keratinocytes were prepared for conversion of OD to number of cells. Raw data of standard curves can be found in Supplemental Fig. 1.

2.8 Statistical Analysis

Data are given as mean $\pm$ standard deviation from n biological repeats as indicated. Statistically significant differences were determined using Student's t test, with $p \leq 0.05$ considered significant.

3 Results

We investigated the effects on human cells caused by direct contact with the materials and the liberation of substances from the materials, as well as the effects on cells caused only by the liberation of substances from the materials. BM-MSCs were cultured either directly on the materials (placed in inserts), or cultured directly on inserts with the materials placed at the bottom of the dish. Keratinocytes could not be tested for the effects caused by direct physical contact with the materials because previous experiments had shown that keratinocytes require collagen-1 coating for their culture (direct culture on either inserts or materials was not successful). Therefore, keratinocytes were seeded on collagen-1-coated bottoms of cell culture dishes and the materials were placed in inserts for the investigation of the effects on cells caused by potential liberation of substances from the materials.

3.1 Numbers of Viable Cells

When skin epidermal keratinocytes (Fig. 2) were cultured with the various materials within the medium but not in

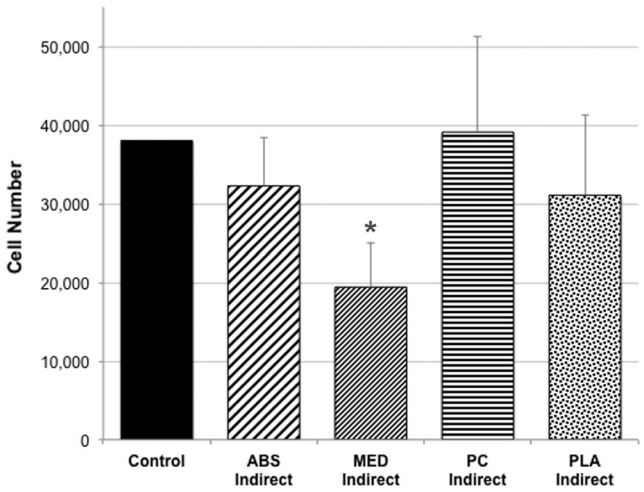

Fig. 2 Numbers of viable keratinocytes. Numbers of cells were analyzed by MTT assay after 48 h in culture on collagen-1-coated dishes, with inserts having discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA), respectively, immersed into cell culture medium. Controls were performed with keratinocytes cultured with empty inserts (no material) immersed into cell culture medium. Data are given as mean ± standard deviation from three biological repeats. Asterisk indicates statistically significant difference relative to control obtained using Student's *t* test (*$p < 0.05$)

direct contact, cell numbers were significantly reduced to 51 % of controls by MED; other materials did not affect cell numbers. BM-MSCs (Fig. 3) in indirect culture with any of the materials in the culture medium (but without direct contact) were not affected significantly in terms of cell numbers. Direct culture on ABS as well as PC

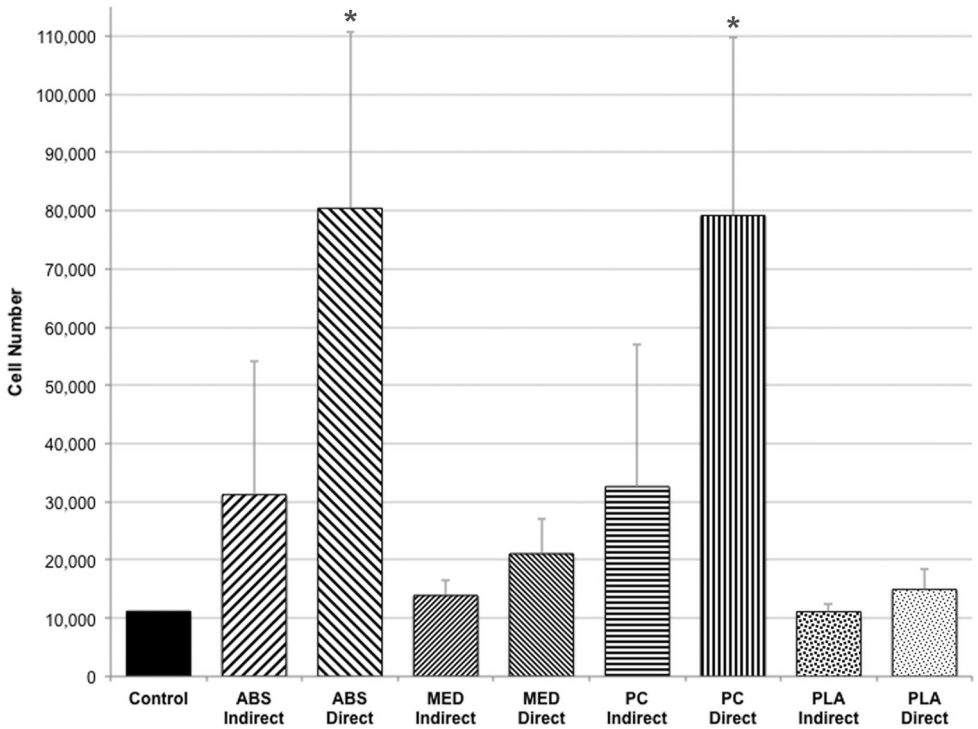

Fig. 3 Numbers of viable bone marrow mesenchymal stromal cells. Numbers of cells were analyzed by MTT assay after 48 h in culture with or on various materials. In indirect culture, cells were cultured on inserts, having discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA), respectively, in cell culture plate bottom well immersed into medium; in direct culture, cells were cultured on discs of either ABS, MED, PC, or PLA, with disks placed in inserts immersed into culture medium. Controls were performed with cells cultured on inserts without any discs. Data are given as mean ± standard deviation from five biological repeats. *Asterisk* indicates statistically significant difference relative to control obtained using Student's *t* test (*$p < 0.05$)

increased cell numbers significantly, about seven-fold when compared to controls. Culture on MED or PLA did not affect cell numbers significantly.

3.2 Cell Imaging

BM-MSCs in direct culture on the materials were analyzed for their attachment to the surface using confocal microscopy (Fig. 4). The nucleic-acid-specific dye DAPI localizes cell distribution by detecting cell nuclei, while the F-actin-specific dye phalloidin indicates cytoskeleton formation. Cells on ABS appeared mostly in aggregates, whereas cells on MED, PC, and PLA were rather evenly distributed throughout the area. This pattern could indicate that initial cell attachment on ABS is impeded. Actin expression was most prominent in cells cultured on PLA, and less noticeable in culture on other materials.

3.3 Surface Topography Analyses

Because surface topography can affect cell attachment, we analyzed the surfaces of the four printed materials (Fig. 5). Among the materials investigated, PC exhibited the smoothest surface. ABS appeared relatively smooth, having some indentations with no sharp edges. PLA presented a uniform uneven surface, which appeared to have only small differences in height. MED demonstrated the roughest surface, having a nonuniform uneven topography with indentations of various sizes.

3.4 Cell Viability

Cell viability was measured by LDH enzyme activity in culture medium samples and given as U/L. Keratinocytes were not affected in terms of their viability by culture with any material compared to controls (Fig. 6). In contrast, BM-MSCs (Fig. 7) demonstrated increased cell death after direct culture on all materials, and also indirect culture with PC and PLA. ABS and MED did not affect cell viability in indirect culture. Because BM-MSCs in direct culture on ABS and PC demonstrated the highest cell numbers after 48 h in culture from all conditions analyzed (Fig. 3), their actual per cell LDH activity was considerably lower compared to that of other cultures, which had fewer cells.

3.5 Glucose Consumption and Lactate Production

Keratinocytes (Fig. 8) in culture with MED consumed significantly higher amounts of glucose and produced more lactate per cell compared to those of controls. Because cultures of keratinocytes with MED also demonstrated significantly lower cell numbers than cultures in control conditions and with other materials, the higher metabolic rates were not due to increased cell proliferation but higher

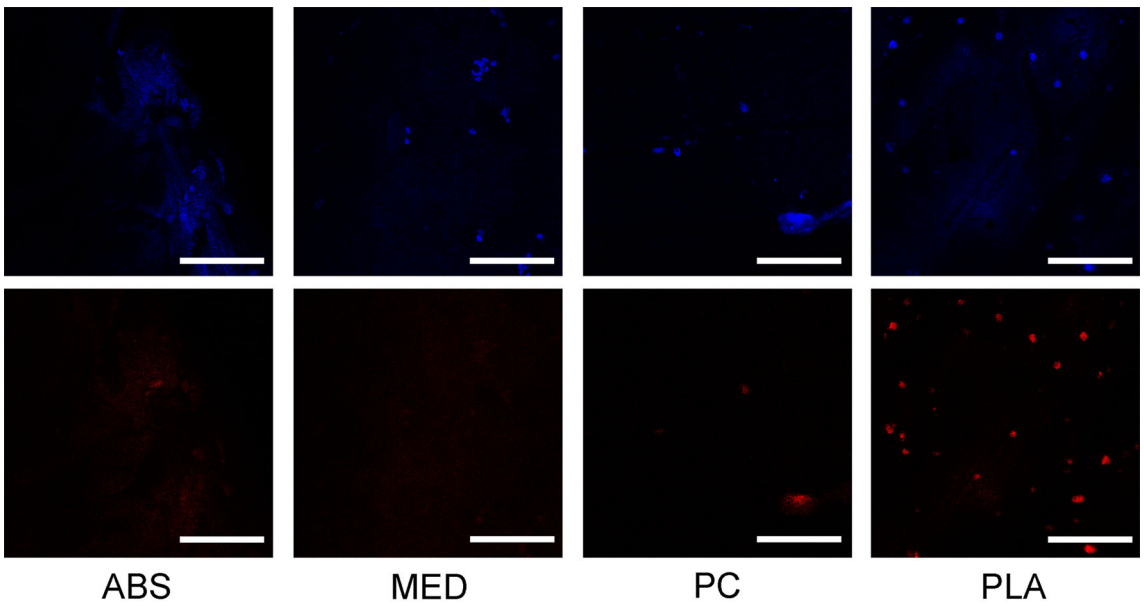

Fig. 4 Cell imaging of bone marrow mesenchymal stromal cells. Distribution of cells and cytoskeleton protein expression after 48 h in culture on various materials was imaged using fluorescence confocal microscopy. Cells were cultured on discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA), respectively. After fixation, cell nuclei were stained with DAPI (*blue*, *upper panel*) and cytoskeleton-specific F-actin was stained with AF568-phalloidin (*red*, *lower panel*). *Scale bar* = 200 μm

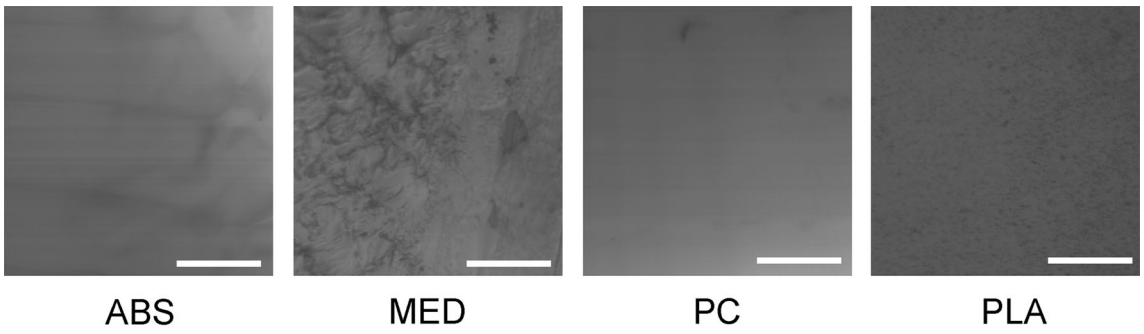

Fig. 5 Material surface topography analyses. Surface topographies of discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA) were investigated using laser scanning microscopy. *Scale bar* = 200 μm

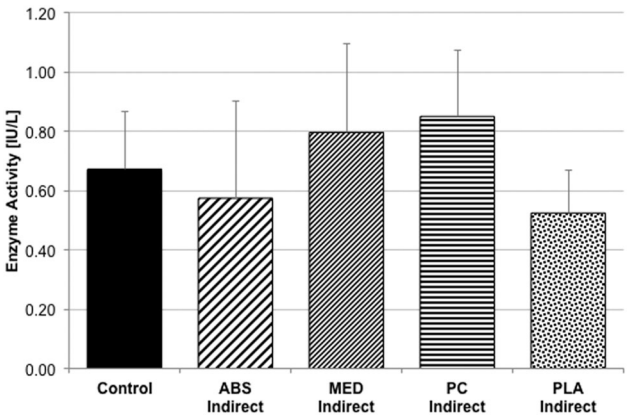

Fig. 6 Cell viability of keratinocytes. Culture media of keratinocytes were analyzed for LDH activity after 48 h in culture on collagen-1-coated dishes, with inserts having discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA), respectively, immersed into cell culture medium. Controls were performed with keratinocytes cultured with empty inserts immersed into cell culture medium. Data are given as mean ± standard deviation from three biological repeats

per cell metabolic rates, indicative of cell differentiation. ABS, PC, and PLA did not change glucose consumption or lactate production.

BM-MSCs (Fig. 9) had the lowest glucose consumption and lactate secretion per cell in direct culture on ABS and PC. In general, glucose consumption and lactate production per cell in culture with or on all materials was statistically significantly reduced, with the exception of cells in indirect culture with PLA. As BM-MSC cultures did not show decreased cell numbers, but rather mostly increased cell numbers, the observed higher metabolic rates can be correlated to increased cell proliferation.

3.6 Gene Expression Analyses

We analyzed the expression of genes specific for cell types and functionality of cultures of keratinocytes (Table 1) and BM-MSCs (Table 2). Ki67 (MKI67) is a proliferation-specific marker. A major function of the transcription factor early growth response 1 (EGR1) is the activation of differentiation-associated genes and the suppression of growth. We were also interested in studying the potential effects of estrogen-like-acting plasticizers that might leach from plastics; therefore, we analyzed the expression of cathepsin D (CTSD), which is an early response gene of estrogen receptor activation. Of further interest was the possible induction of intrinsic (apoptosis) or extrinsic (necrosis) mediated cell death caused by the culture with or on the materials, and thus we measured the expression of apoptosis marker caspase 3 (CASP3) and tumor necrosis factor alpha (TNF). Keratinocyte-specific markers included various keratins (KRT) and alpha-6-integrin (ITGA6). The diverse keratins of the skin epidermis have been associated with different developmental stages and functionality [10]. Embryonic differentiation is accompanied by expression of KRT13; this developmentally induced differentiation can be mimicked in vitro by the addition of retinoid acid [11]. KRT13 expression in adult skin is implicated to be a re-induction of the embryonic form of differentiation [12, 13]. Progenitor basal cells of the epidermis express the major pair of keratins, KRT5 and 14, and lower quantities of KRT15 protein [14–16]. During differentiation, keratinocytes turn off KRT5/14 gene expression [17, 18] (while the proteins are still expressed). KRT15 can be found in co-expression with KRT5/14 in basal keratinocytes, the bulge of hair follicles, and suprabasal epidermis. The role of KRT15 is somewhat under debate, and its regulation by two different signaling pathways and exact role depend on the epidermal cell layer, as reviewed by Bose et al. [19]. In the basal layer, KRT15 has been used as a stem cell marker, whereas within the suprabasal layer, KRT15 expression has been associated with differentiation. BM-MSCs are typically positive for CD90 (THY1). Further, BM-MSCs have been demonstrated to possess multilineage differentiation potential, which can be

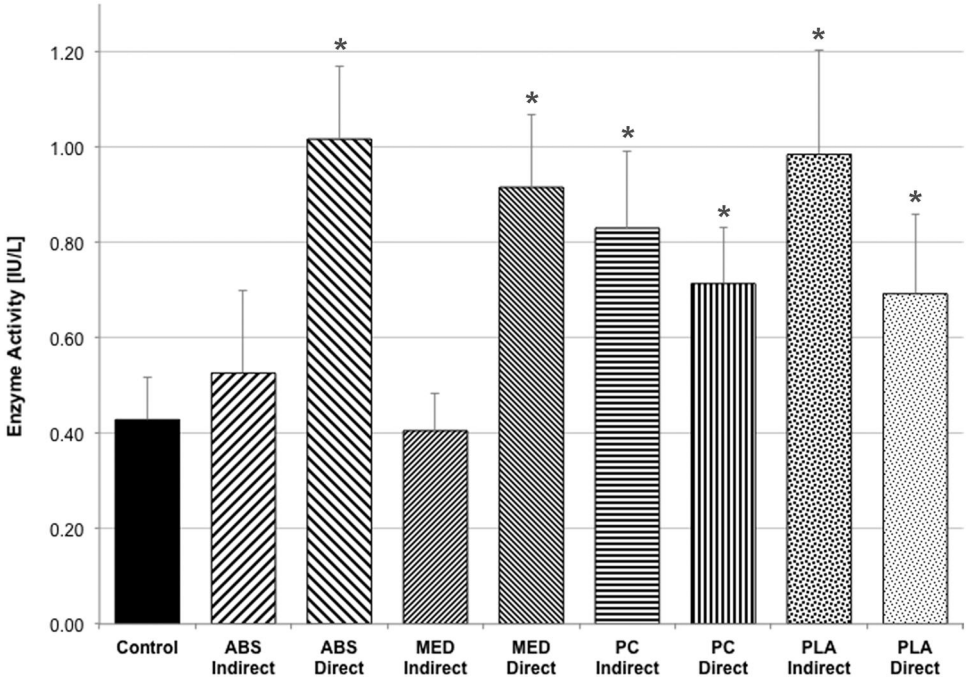

Fig. 7 Cell viability of bone marrow mesenchymal stromal cells. Cell culture media were analyzed for LDH activity after 48 h in culture. In indirect culture, cells were cultured on inserts, having discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA), respectively, in cell culture plate bottom well immersed into medium; in direct culture, cells were cultured on discs of either ABS, MED, PC, or PLA, with disks placed in inserts immersed into culture medium. Controls were performed with cells cultured on inserts without any discs. Data are given as mean ± standard deviation from five biological repeats. Asterisk indicates statistically significant difference relative to control obtained using Student's *t* test ($*p < 0.05$)

induced under specific culture conditions. Therefore, in order to investigate potential differentiation towards certain lineages caused by the materials, we determined the expression of chondrogenic-, osteogenic-, and adipogenic-lineage-specific markers, represented by lineage-specific chondrocyte marker aggrecan (ACAN), osteocyte marker bone gamma-carboxyglutamate protein (BGLAP) (also known as osteocalcin), and adipocyte marker adiponectin (ADIPOQ), respectively.

We found that skin keratinocytes demonstrated a material-dependent response on gene expression (Table 1). In general, PC did not elicit any significant changes in the expression of the genes analyzed, and PLA only increased the expression of Keratin-15, the epithelial intermediate filament protein that is abundantly expressed in the basal layer of adult epidermis. In contrast, ABS and MED showed similar and significant effects on the expression of several genes. The proliferation-specific marker Ki67 was reduced to less than 40 % compared to controls. Simultaneously, the expressions of the EGR1, Keratin-15, and CTSD genes were up-regulated. Apoptosis or necrosis did not seem to be induced by any material as the expression of neither CASP3 nor TNF was significantly changed. This indicates that, even without direct contact, in keratinocytes, both MED and ABS suppress proliferation and growth, induce differentiation, and possibly release estrogen-like substances, as indicated by induced CTSD expression.

BM-MSCs in indirect culture demonstrated a lower response in gene expression compared to that of keratinocytes when cultured with the materials indirectly (Table 2). In culture with MED and PLA, CTSD expression was up-regulated. PLA also increased slightly, but statistically significantly, the expression of apoptosis marker CASP3 and mesenchymal marker THY1. The chondrogenic lineage differentiation marker aggrecan was reduced in culture with ABS and PC. When BM-MSCs were cultured directly on the materials, significantly strong changes in the expression of genes were observed. CTSD, EGR1, and CASP3 were up-regulated by all materials, with MED and ABS showing the strongest and second strongest effects, respectively, on CTSD and EGR expression. MED up-regulated EGR1 expression more than ten-fold, and MED was the only material that significantly reduced the expression of proliferation marker Ki67. The mesenchymal marker THY1 was slightly up-regulated by all materials (however, in culture on ABS, this up-regulation was not statistically significant). As in indirect culture, ABS in direct culture reduced the expression of chondrogenic marker aggrecan, but increased that of the osteogenic marker osteocalcin (BGLAP). In general, none of the

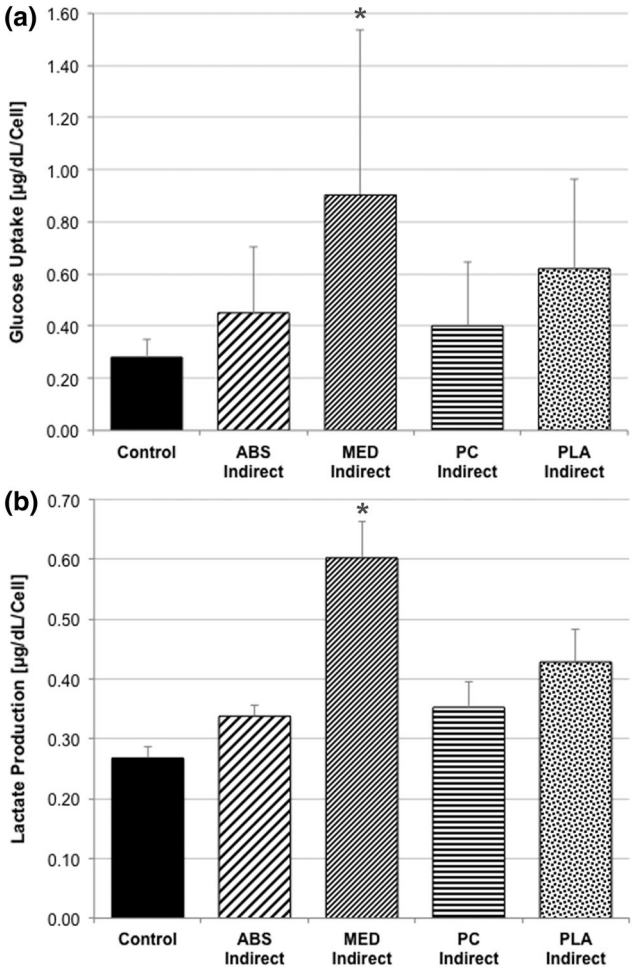

Fig. 8 Metabolic activity of keratinocytes. **a** Glucose consumption and **b** lactate production per cell were measured in cell culture media after 48 h in culture with inserts having discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA), respectively, immersed into cell culture medium. Controls were performed with keratinocytes cultured with empty inserts immersed into cell culture medium. Data are given as mean ± standard deviation from three biological repeats. *Asterisk* indicates statistically significant difference relative to controls obtained using Student's *t* test (*$p < 0.05$)

cultures, including controls, expressed adipogenic-lineage-specific marker adiponectin or TNF.

4 Discussion

In this study, we compared the response of ectodermal and mesodermal primary human cells to four materials used in regenerative medicine, tissue engineering, and thermal 3D printing. Current advances in additive manufacturing (3D printing) allow the layer-by-layer creation of sophisticated in vivo-like structures (for reviews, see [20–22]); for example, much progress has been achieved in cartilage and bone tissue engineering by the ability to print interconnected porous polymer networks, to implement hydrogels, and to print simultaneously different scaffolds and cells in order to recreate complex tissue-like structures. Yet, the use of non-biological materials for such structures requires the evaluation of their biocompatibility.

In the present study, we found that the investigated materials elicited different responses in both cell types, with MED and ABS having the strongest effects. Gene expression analyses suggest that cell proliferation was reduced via decreased expression of proliferation-specific gene MK67 and increased expression of EGR1, which activates differentiation-specific genes and the suppression of growth. In direct culture, all materials induced apoptosis in BM-MSCs via the induction of CASP3. Expression of caspases has been associated traditionally with the induction of apoptosis, and emerging evidence has demonstrated a role of caspases in cell and tissue differentiation [23]. CASP3 has been shown to be essential for BM-MSC differentiation in vitro and in vivo by influencing the TGF-β/Smad2 pathway and cell cycle progression [24]. BMP-4-induced osteoblasts [25] demonstrated the activation of caspases (including CASP3) without apparent induction of apoptosis or necrosis.

MED caused the strongest up-regulation of CTSD, an early estrogen response gene [26], in all cultures, indicating that plasticizers (such as bisphenol A and its derivatives) acting as estrogen-like substances [27] could leach out from this material and affect the cells. MED also significantly reduced the cell numbers of keratinocytes, even when not in direct contact with the cells (Fig. 1). Similar to ABS, gene expression analyses of keratinocytes demonstrated significantly reduced gene expression of the proliferation marker protein Ki67, and simultaneous induction of keratinocyte differentiation-specific genes KRT15 and 13 (although increased KRT13 expression was not statistically significant). Keratinocyte differentiation due to MED is also supported by the facts that cultures showed significantly increased metabolism (glucose uptake and lactate production), thereby having the lowest cell numbers, i.e., a considerably high per cell metabolic rate. LDH activity, an indicator of cell death, was not significantly increased in keratinocyte cultures that had no direct contact with MED. These results indicate that substances released from MED lead to reduced cell proliferation and induce differentiation, but do not induce cell death; in direct contact, however, MED also induces cell death via the induction of CASP3. MED is a thermoplastic for use in medical devices such as hearing aids, as well as dental and orthodontic models. The composition of this proprietary material is not available; according to the manufacturer, this material has been tested for biocompatibility and has medical approval. Studies using MED in cell culture have

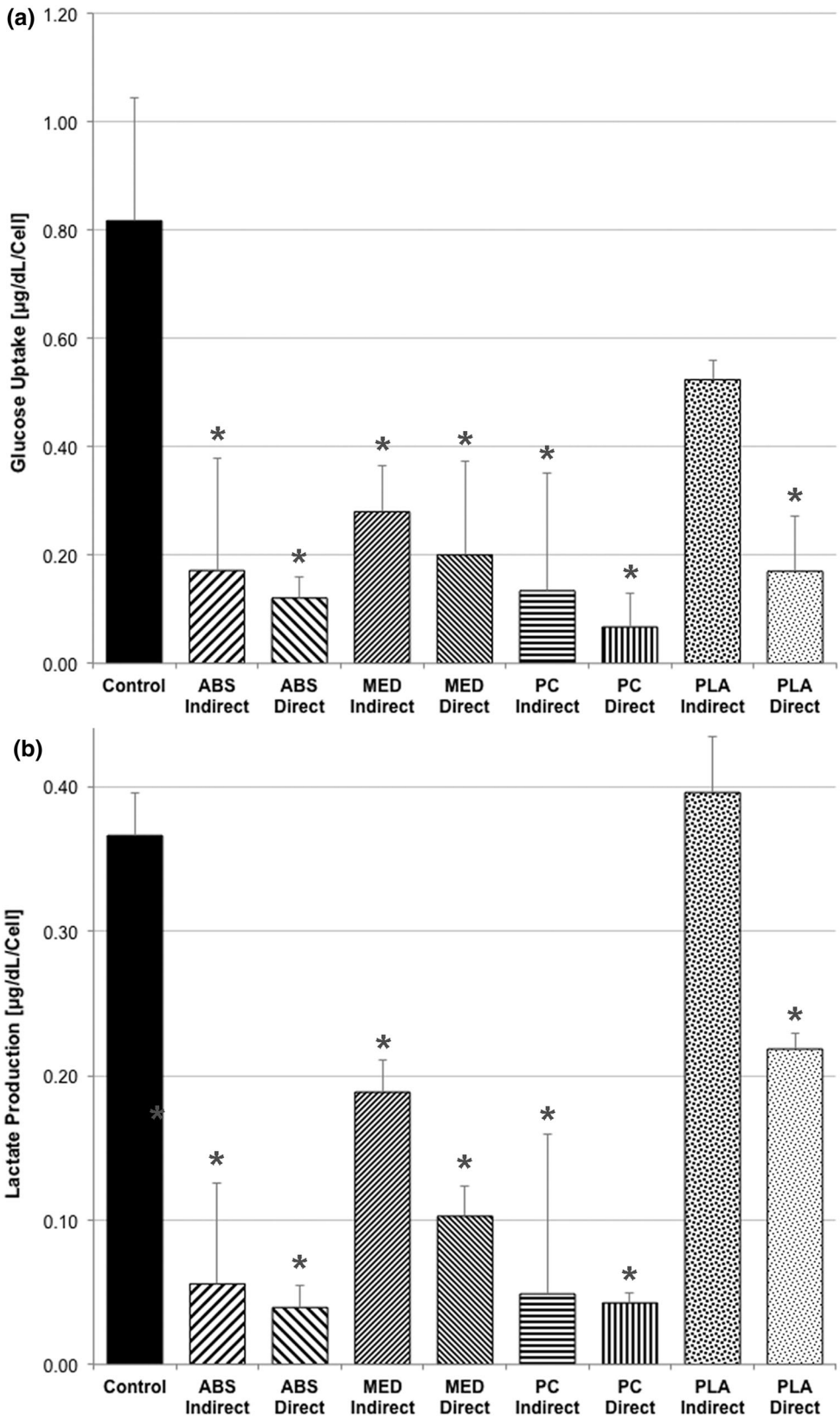

Fig. 9 Metabolic activity of bone marrow mesenchymal stromal cells. **a** Glucose consumption and **b** lactate production per cell were measured in cell culture media after 48 h in culture. In indirect culture, cells were cultured on inserts, having discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA), respectively, in cell culture plate bottom well immersed into medium; in direct culture, cells were cultured on discs of either ABS, MED, PC, or PLA, with disks placed in inserts immersed into culture medium. Controls were performed with cells cultured on inserts without any discs. Data are given as mean ± standard deviation from five biological repeats. *Asterisk* indicates statistically significant difference relative to controls obtained using Student's *t* test ($*p < 0.05$)

not been published, but based on the described properties, MED seems to be a quite promising material for tissue engineering applications. The findings of the present study somewhat caution the use of this material in cell culture or bioengineering applications with direct prolonged contact with cells.

Table 1 Gene expression analyses of keratinocytes

	Indirect Culture				
Gene	**Control**	**ABS**	**MED**	**PC**	**PLA**
MK67	100	35* ± 29	39* ± 37	67 ± 43	89 ± 39
CTSD	100	243* ± 116	319* ± 209	138 ± 56	127 ± 56
EGR1	100	281* ± 160	224** ± 25	101 ± 12	107 ± 32
CASP3	100	83 ± 34	102 ± 44	94 ± 31	93 ± 25
TNF	100	66 ± 27	90 ± 36	75 ± 20	69 ± 26
KRT5	100	76 ± 24	111 ± 19	95 ± 10	98 ± 9
KRT13	100	152 ± 84	168 ± 95	118 ± 80	127 ± 96
KRT14	100	101 ± 8	130 ± 23	104 ± 13	96 ± 13
KRT15	100	399* ± 178	674** ± 117	167 ± 64	228** ± 33
ITGA6	100	77 ± 35	138 ± 42	108 ± 8	107 ± 8

Human adult epidermal keratinocytes were cultured for 48 h on collagen-1-coated dishes, with inserts having discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA), respectively, immersed into cell culture medium. Controls were performed with keratinocytes cultured with empty inserts immersed into cell culture medium. Gene expression was normalized to controls (set as 100 %), and analyzed for proliferation marker Ki67 (MKI67), cathepsin D (CTSD), early growth response 1 (EGR1), caspase 3 (CASP3), tumor necrosis factor alpha (TNF), keratins (KRT) 5, 13, 14, and 15, and alpha-6 integrin (ITGA6). Data are given in percent and are means from three biological repeats ± standard deviation. Asterisks indicate statistically significant differences relative to control obtained using Student's *t* test (* $p < 0.05$; ** $p < 0.01$), highlighting significant down-regulation with light grey fill and up-regulation with dark grey fill

ABS is not often used in tissue engineering, but has been used for molding to create 3D cellular shapes of physiological structures such as ears [6] and tympanic membranes [7]. In these studies, however, the biocompatibility of ABS was not investigated. Notably, we found that ABS up-regulated the osteogenic gene osteocalcin (BGLAP) but down-regulated the chondrogenic gene aggrecan in BM-MSCs in direct culture, indicating that certain materials support a specific lineage direction. This seems to be an important factor to be aware of when specific lineage direction is the goal. Our data indicate that ABS does not support chondrogenic lineage differentiation, but rather

Table 2 Gene expression analyses of bone marrow mesenchymal cells

Indirect Culture					
Gene	**Control**	**ABS**	**MED**	**PC**	**PLA**
MKI67	100	84 ± 37	79 ± 29	93 ± 38	101 ± 38
CTSD	100	118 ± 10	153* ± 20	122 ± 16	122** ± 2
EGR1	100	115 ± 7	123 ± 11	117 ± 13	134 ± 37
CASP3	100	105 ± 32	118 ± 20	107 ± 17	110* ± 8
TNF	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
THY1	100	116 ± 36	115 ± 36	115 ± 19	118* ± 9
ACAN	100	59** ± 11	68 ± 29	75** ± 8	101 ± 25
BGLAP	100	111 ± 22	120 ± 27	98 ± 2	89 ± 26
ADIPOQ	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Direct Culture					
Gene	**Control**	**ABS**	**MED**	**PC**	**PLA**
MKI67	100	69 ± 32	58* ± 37	111 ± 48	97 ± 40
CTSD	100	231** ± 23	657* ± 231	199** ± 14	172* ± 26
EGR1	100	514* ± 269	1066* ± 644	364* ± 155	323* ± 150
CASP3	100	171* ± 51	285* ± 179	176* ± 54	161* ± 34
TNF	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
THY1	100	141 ± 42	131* ± 19	154* ± 38	155* ± 34
ACAN	100	46* ± 41	79 ± 82	121 ± 153	93 ± 88
BGLAP	100	171* ± 50	150 ± 119	136 ± 46	113 ± 30
ADIPOQ	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Human adult bone marrow mesenchymal stromal cells were cultured for 48 h. In indirect culture, cells were cultured on inserts, having discs of acrylonitrile butadiene styrene M30i (ABS), PolyJet photopolymer MED610 (MED), polycarbonate-ISO (PC), and polylactic acid (PLA), respectively, in cell culture plate bottom well immersed into medium; in direct culture, cells were cultured on discs of either ABS, MED, PC, or PLA, with disks placed in inserts immersed into culture medium. Controls were performed with cells cultured on inserts without any discs. Gene expression was normalized to controls (set as 100 %), and analyzed for proliferation marker Ki67 (MKI67), cathepsin D (CTSD), early growth response 1 (EGR1), caspase 3 (CASP3), tumor necrosis factor alpha (TNF), Thy-1 cell surface antigen (THY1), aggrecan (ACAN), bone gamma-carboxyglutamate protein (BGLAP), and adiponectin (ADIPOQ). Data are given as percent and are means from four biological repeats ± standard deviation. Asterisks indicate statistically significant differences relative to control obtained using Student's t test (* $p < 0.05$; ** $p < 0.01$), highlighting significant down-regulation with light grey fill and up-regulation with dark grey fill

osteogenic lineage differentiation, suggesting that alternative materials should be used for chondrogenic differentiation. Also, direct cultures of BM-MSCs on ABS resulted in significantly higher cell numbers compared to controls, indicating that ABS can support cell growth and attachment. A comparative environmental toxicity study of various plastics, including ABS, polypropylene, polyethylene, polyvinyl chloride, and epoxy, demonstrated that ABS had the least acute toxicity of the materials investigated on the water flea *Daphnia magna* [28].

In the present study, PC demonstrated the lowest effects of all materials on both cell types investigated. Similar to ABS, the direct culture of BM-MSCs on PC resulted in significantly higher cell numbers than controls, indicating that PC can promote cell growth. The expression of the proliferation-associated gene MK67 was not significantly up-regulated in these cultures, suggesting that the increased cell numbers could be due to either increased initial cell attachment or induction of other proliferation-relevant genes that were not the subject of our studies, for example cyclins. A study using the NIH 3T3 mouse fibroblast cell line in orthodontic bracket metallic materials found that PC caused the highest cytotoxicity compared to those of monocrystalline ceramics, nickel-containing metals, nickel-free metals, and polycrystalline ceramic brackets [3]. These findings might indicate that metals and ceramics demonstrate somewhat more biological inert properties than do plastics. A recent study using cell lines focused on the role of bisphenol A leaching from PC [29]; it was demonstrated that the metabolic activity of sensitive cell lines can be influenced by bisphenol A. In the present study, we did not observe a significant up-regulation of CTSD in indirect cultures, but did in direct culture of BM-MSCs on PC. This effect, however, was also observed in culture on other materials and was much stronger for MED.

PLA is a biodegradable material often used for tissue engineering [1]. For example, PLA-based silver-ions-including nanofibers with anti-microbial properties were tested successfully in vitro for the development of skin wound dressings [30]. In a recent study by Borowiec et al. [31], a 3D scaffold was created from PLA, which demonstrated better biocompatibility and higher albumin secretion (a mature, differentiated hepatocyte function) of the hepatic cell line HepG2 compared to those obtained with PC scaffolds. We observed higher cell numbers and lower metabolic activity in cultures of BM-MSCs on PC than those for PLA, indicative of PLA inducing more differentiation. These findings are consistent with the trends observed by Borowiec et al. for HepG2 cells. In the present study, we also found that PLA exerted lower effects on gene expression of keratinocytes than did MED or ABS. Although cell viability in indirect culture of keratinocytes was not affected, BM-MSCs showed increased cell death in direct as well as indirect cultures with PLA. Because an increase in cell death was observed (as indicated by increased LDH activity as well as CASP3 gene expression) even though cell numbers after 48 h in direct culture were similar to controls, it seems likely that the initial cell attachment rates of BM-MSCs to PLA and MED were higher than that for controls, compensating for the loss of cells during culture. BM-MSCs cultured directly on PLA demonstrated the most prominent expression of F-actin. Actin is the major component of the cytoskeleton, providing not only an intracellular scaffold but also connections to the extracellular matrix through focal adhesion points [32]. PLA thus provided the best support of the intracellular scaffold as well as extracellular adhesion.

The surface topography of cell culture materials has been shown to play an important role in cell attachment and functionality [33, 34]. It has to be considered that 3D printing could result in microscopically different surface topographies than those obtained during commercial manufacturing of cell culture devices such as Petri dishes. We observed that PC had the smoothest surface and MED the roughest. Interestingly, Herath et al. [35] investigated the response of osteoblasts to zirconia ceramic surfaces, and found that although the more uneven surfaces provided more initial attachment, the overall cell proliferation and numbers (measured as DNA yield) were highest on the more polished surfaces. These results are consistent with our observations, in which the smoothest surfaces of PC and ABS demonstrated the highest cell numbers of BM-MSCs in direct culture on the materials.

5 Conclusion

In conclusion, our results indicate that PC and PLA have better biocompatibility than that of MED and ABS, which significantly affected adult human primary cell types. Taken together, structural components for use in regenerative medicine, tissue engineering, and 3D printing exhibit specific effects on primary ectodermal and mesodermal cell types, which suggests that careful consideration should be taken of the cell source and materials to be used for a specific purpose.

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Conflict of interest The authors declare that they have no conflicts of interest.

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