Coculture Effects on the Osteogenic Differentiation of Human Mesenchymal Stromal Cells

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Cell-based bone regeneration is generally pursued based on single cell type approaches, for which human adipose tissue-derived mesenchymal stromal cells (AT-MSCs) are frequently used, owing to their easy accessibility and relatively large yield. In view of multiple cell types involved in physiological bone regeneration, this study aimed to evaluate the osteogenic differentiation of AT-MSCs upon co-culture with endothelial cells or macrophages in a direct or indirect *in vitro* co-culture set-up. Our hypotheses were that 1) endothelial cells and macrophages stimulate AT-MSCs proliferation and osteogenic differentiation and that 2) these two cell types will more profoundly affect osteogenic differentiation of AT-MSCs in a direct compared to an indirect co-culture set-up, because of the possibility for both cell-cell interactions and effects of secreted soluble factors in the former. Osteogenic differentiation of AT-MSCs was stimulated by endothelial cells, particularly in direct co-cultures. Although initial numbers of AT-MSCs in co-culture with endothelial cells were 50% compared to monoculture controls, equal levels of mineralization were achieved. Macrophages showed a variable effect on AT-MSCs behavior for indirect co-cultures and a negative effect on osteogenic differentiation of AT-MSCs in direct co-cultures, the latter likely due to species differences of the cell types used. The results of this study demonstrate potential for cell combination strategies in bone regenerative therapies.

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INTRODUCTION

One of the challenges confronted by orthopedic surgeons is to repair large segmental bone defects caused by trauma, infection, tumor resection, skeletal bone abnormalities or other bone disorders. So far, such clinical challenges have been predominantly approached using autologous bone grafts. However, this approach is accompanied by multiple limitations, e.g., quantity and quality issues of autologous donor bone and issues related to a second surgery site [1]. Alternative treatment options have been explored using the toolbox of tissue engineering (i.e., scaffolds, biologicals and cells). In view of cell-based strategies, major emphasis has focused on mesenchymal stromal cells (MSCs) [2]. These MSCs can be obtained from various tissues, including bone marrow (BM), skin, muscle and adipose tissue (AT).

However, the yield of MSCs usually varies within these different tissues [3]. In bone regenerative research, the most commonly used cell types are BM derived MSCs but the disadvantages of those cell are related to the invasive extraction methods and limited cell yield [4,5]. In contrast to BM-MSCs, substantial numbers of AT-MSCs can be harvested from fat tissues, with less morbidity and easy accessibility [6]. Moreover, studies indicate that AT-MSCs and BM-MSCs in co-culture with human umbilical vein endothelial cells (HUVECs) have shown similar potential for osteogenic differentiation and mineralization [6-8].

To develop cell-based bone tissue regenerative strategies, it is necessary to understand natural bone regeneration and fracture healing processes. Bone is a highly vascularized tissue with self healing, regeneration and remodeling ability, representing biological processes that involve many cell types [9]. Vascularization is an essential process during bone fracture healing, regeneration and remodeling. Fracture healing starts with an inflammatory phase, followed by reparative and remodeling phases. During inflammation, white blood cells (i.e., monocytes, macrophages, neutrophils, and lymphocytes) accumulate

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at the defect site, where they form granulation tissue [10]. Next, MSCs and endothelial cells migrate to the defect site, where they promote vascularization and bone repair. In this phase, many signaling mechanisms evoke subsequent differentiation of MSCs into osteoblast and formation of osteoclasts from hematopoietic precursor cells. Fracture healing is completed with a bone remodeling phase, during which bone retains its original shape, structure, and mechanical strength [10]. Consequently, bone regeneration and fracture healing are complex physiological processes, in which many cell types (i.e., MSCs, endothelial cells, macrophages) and multiple factors (signaling pathways) are involved, which coordinate cell migration, cell proliferation, osteogenic differentiation and extracellular matrix formation [11]. MSCs from different origin co-cultured with endothelial cells already received huge interest in bone regenerative research, and it was proven that endothelial cells promote mineralization of MSCs *in vitro* and bone formation *in vivo* [12,13]. Moreover, cellular crosstalk between MSCs and endothelial cells activates adequate osteogenic signals aided by the expression of signaling factors [14] (e.g., vascular endothelial growth factor), which is shown to be essential for bone repair [15]. Several studies indicate that macrophages also play a crucial role in bone regeneration, because they are involved in extracellular matrix formation [16,17] and secrete a plethora of signaling molecules that are linked to multiple processes during osteogenesis [18,19]. In view of this, it is straightforward to study the behavior of AT-MSCs in *in vitro* culture conditions based on co-culture with endothelial cells or macrophages [14].

Different studies, which focused on cell-cell interactions in a direct or indirect co-culture set-up [20–22], showed that direct cell-cell contact has different effects on cellular communication compared to indirect contact [23,24]. This indicates that cell co-culture set-up plays an important role in cellular communication [20]. MSCs in direct contact with endothelial cells showed positive effects regarding osteogenic differentiation in comparison to indirect contact [12,25]. It has been also proven that direct cellular contact between MSCs and adult cardiomyocytes promotes MSC differentiation into cardiomyocytes, in contrast to indirect contact. Moreover, in an indirect co-culture set-up, signaling molecules (growth factors), which were secreted in the culture medium, showed no effect on MSC differentiation [23]. Consequently, it is important to understand what mechanisms are involved in direct and indirect cell-cell communication [26,27].

This study aimed to evaluate the osteogenic differentiation of AT-MSCs upon co-culture with endothelial cells or macrophages in a direct or indirect co-culture set-up. Our hypotheses were that 1) endothelial cells and macrophages stimulate AT-MSCs proliferation and osteogenic differentiation, and that

2) these two cell types will more profoundly affect osteogenic differentiation of AT-MSCs in a direct compared to an indirect co-culture set-up, because of the possibility for both cell-cell interactions and effects of secreted soluble factors.

MATERIALS AND METHODS

Cell culture

AT-MSCs were isolated from subcutaneous fat tissue of three different healthy female human donors. After institutional approval (IRB no. 2010-00561) and written informed consent, fat tissue was obtained from the Department of Plastic Surgery (Radboudumc, Nijmegen, the Netherlands) as discarded tissue from breast reconstructive surgery. AT was harvested from abdomen, after resection and stored in phosphate buffered saline (PBS; B. Braun, Melsunge, Germany) before cell isolation. The procedure of AT-MSCs isolation is described in detail elsewhere [6,12,28,29]. Briefly, AT was minced with a surgical scalpel in very small pieces, washed with PBS and digested with 0.1% collagenase A (Roche Diagnostics; Mannheim, Germany)/1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA)/PBS at 37°C for 45 min. Afterwards, digested tissue was filtrated with a 0.2 µm filter, and cells were centrifuged using a Ficoll density centrifugation method. Harvested cells were counted and expanded in proliferation medium, consisting of alpha minimal essential medium (Gibco®, Life Technologies, Grand Island, NE, USA), supplemented with 5% platelet lysate at 37°C in humid atmosphere with 5% CO₂ (the complete composition of proliferation media is given in Table 1). Medium was changed twice a week. Cells were passaged upon reaching ~80% confluency using 0.25% w/v trypsin/0.02% EDTA (Gibco®). After the first passage, cells were examined by fluorescence-activated cell sorting (FACS) for positive expression of CD73 and CD105 (eBioscience, San Diego, CA, USA) and negative expression of CD45 (R&D system, Abingdon, UK).

As endothelial cells, HUVECs were obtained from a commercial source (Becton Dickinson Biosciences, BD, Breda, the Netherlands). Cells were expanded at 37°C in proliferation medium consisting of Medium 200 (Gibco®), supplemented with low serum growth supplement kit (Gibco®), in humid atmosphere with 5% CO₂. medium was changed twice a week. Cells were passaged upon reaching ~80% confluency using 0.25% w/v trypsin/0.02% EDTA.

As a monocyte/macrophage cell type, RAW 264.7 cells were used, which were obtained from a commercial source (Sigma-Aldrich). RAW 264.7 cells were expanded in proliferation medium consisting of Dulbecco's Modified Eagle Medium (Gibco®), supplemented 2 mM L-glutamine and 10% fetal bovine serum (Lonza, Basel, Switzerland). Medium was refreshed

twice a week and cells were passaged upon reaching ~80% confluency.

Experimental co-cultures

For the direct co-culture set-up, AT-MSCs were cultured with either HUVECs or RAW 264.7 cells in a 1:1 cell ratio, with total number of 10.000 cells/cm² (24 well plates/n=3). As a control, AT-MSC monoculture (10.000 cells/cm²) was used (Fig. 1). The experimental groups were following: direct AT-MSCs in monoculture as a control (AT-AT), AT-MSCs co-cultured with HUVECs (AT-HU), and AT-MSCs co-cultured with Raw 264.7 cells (AT-RAW).

For the indirect co-culture set-up, AT-MSCs were cultured in 24 well plates, which were combined with transwell inserts (Greiner Bio-One, Kremsmünster, Austria), into which HUVECs or RAW 264.7 cells were cultured. The cell seeding density in 24 well plates was 10.000 cells/cm² (n=3) and in transwells, the same number of cells were seeded to maintain a 1:1 cell ratio. As a control, AT-MSCs were co-cultured with AT-MSCs in transwells. Experimental groups were following: in-

direct AT-AT (AT-MSCs co-cultured with AT-MSCs), AT-HU and AT-RAW. For all co-culture groups, the same osteogenic medium was used (Table 1).

Cell morphology

During the entire culture period, cell morphology was monitored with an inverted light microscope (Leica DM-IL, 5 W LED Illumination, Rijswijk, the Netherlands) for both direct and indirect co-cultures. Additionally, cell morphology was monitored with scanning electron microscopy (SEM; JEOL 6330F, Peabody, MA, USA), for which samples were prepared at selected time points by washing twice with PBS, fixing with 2% glutaraldehyde for 10 minutes, and washing with 0.1 M Na-cacodylate buffer for 10 minutes. For dehydration, samples were treated with a graded series of ethanol, each for 5 minutes. Finally, samples were incubated in air flow overnight by adding one drop of tetramethylsilane to each sample for complete dehydration. Prior to observation using a SEM, samples were coated with a gold layer.

Figure 1. Overview of co-culture systems of AT-MSCs. AT-MSCs: adipose tissue-derived mesenchymal stromal cells, HUVECs: human umbilical vein endothelial cells.

Table 1. Composition of the proliferation and osteogenic media

Proliferation media	Osteogenic media
Alpha minimal essential medium	Alpha minimal essential medium
5% platelet lysate	5% platelet lysate
10 U/mL heparin	0.2 mM L-ascorbic acid 2-phosphate (Vit C)
100 U/mL penicillin	2 mM L-glutamine
10 µg/ mL streptomycin	100 U/mL penicillin
	10 µg/mL streptomycin
	10 ⁻⁸ M dexamethasone
	0.01 M β-glycerophosphate
	10 U/mL heparin

Table 2. Overview of co-culture results of AT-MSCs from 3 donors compared to respective AT-MSCs (mono-cultures)

	AT-HU		AT-RAW	
	Direct	Indirect	Direct	Indirect
Proliferation				
Donor 1	0	0	+	0
Donor 2	0	0	+	0
Donor 3	0	0	+	0
Differentiation				
Donor 1	+	+	-	+
Donor 2	+	+/-	-	-
Donor 3	+	+/-	-	-
Mineralization				
Donor 1	+	+	-	+
Donor 2	+	-	-	-
Donor 3	+	0	-	-

0: no differences in proliferation, differentiation or mineralization of experimental groups compared to AT-MSCs mono-culture, +: increased proliferation, differentiation or mineralization of experimental groups compared to AT-MSCs mono-culture control, -: decreased proliferation, differentiation or mineralization of experimental groups compared to AT-MSCs mono-culture control, +/- variable proliferation, differentiation or mineralization in experimental groups compared to AT-MSCs mono-culture control. AT-MSCs: adipose tissue-derived mesenchymal stromal cells

Table 3. Overview of direct versus indirect co-culture results with AT-MSCs from 3 human donors

	AT-HU	AT-RAW
	Direct versus indirect co-culture	Direct versus indirect co-culture
Proliferation		
Donor 1	0	-
Donor 2	+	-
Donor 3	+	-
Differentiation		
Donor 1	+	+
Donor 2	-	+
Donor 3	-	+
Mineralization		
Donor 1	-	+
Donor 2	-	+
Donor 3	-	+

0: no differences in proliferation, differentiation or mineralization of experimental groups compared to AT-MSCs mono-culture, +: increased proliferation, differentiation or mineralization of experimental groups compared to AT-MSCs mono-culture control, -: decreased proliferation, differentiation or mineralization of experimental groups compared to AT-MSCs mono-culture control. AT-MSCs: adipose tissue-derived mesenchymal stromal cells

Cell behavior

Cell behavior was analyzed with different biochemical assays, which were used as described previously [12]. Cellular

DNA content was used as a measure for cell proliferation and quantified with the QuantiFluor® dsDNA kit (Promega, Madison, WI, USA). To determine early osteogenic differentiation, alkaline phosphatase (ALP) activity was measured using a colorimetric assay (Sigma-Aldrich) and normalized to cellular DNA content. Finally, for late stage osteogenic differentiation, calcium deposition was measured using a p-nitrophenyl phosphate colorimetric assay (Sigma-Aldrich).

Samples from cell culture experiments were taken at the days 7, 14, 21, 28, and 35. For sampling, cell layers were washed twice with PBS, and stored with 1 mL MilliQ at -80°C. After two repetitive freeze/thaw cycles, samples were used as per instructions of the manufacturer of the assay kits. Sampling for calcium deposition, measurements was performed by incubating the remaining extracellular matrix in the 24 well plates overnight at room temperature with 1 mL 0.5 N acidic acid on a shaking plate. For direct co-culture, the ALP and calcium deposition data were normalized with initial AT-MSCs cell seeding number.

Statistical analysis

The data was statistically analyzed using Graphpad Prism software (GraphPad Software Inc., La Jolla, CA, USA). An one-way ANOVA with post-hoc Dunett's comparisons test was used to analyze the effect of different cell types on proliferation, differentiation and mineralization. An unpaired t-test was used to analyze the data between different co-culture set-up, i.e.,

direct versus indirect. Differences were considered as significant at $p < 0.05$.

RESULTS

This study is based on results using AT-MSCs of three different human donors. A comparative overview of co-culture results compared to simultaneous respective AT-MSCs monoculture controls is presented in Table 2. Similarly, a comparative overview of direct versus indirect co-culture results is presented in Table 3.

Cell morphology and characterization of AT-MSCs

The results of FACS analysis showed that harvested cells were 99% positive for expression of surface markers CD73 and CD105, and completely negative for CD45 expression (data not shown). With inverted light microscopy, it was possible to monitor the cell morphology of all experimental groups. The results at days 7 and 35 for both direct and indirect culture set-up are presented in Figures 2 and 3. Already at day 7, cells completely covered

the culture area of 24 well plates in both direct and indirect culture set-up. Detectable morphological differences were observed also in inserts (indirect culture set-up), especially for AT-MSCs versus RAW 264.7 cells. AT-MSCs showed an elongated and spindle shape morphology, whereas RAW 264.7 cells appeared round shape. At day 35, dense cellular accumulations were observed for all groups, in which mineral depositions (black spots; highlighted with red arrows) were detectable (Figs. 2 and 3).

SEM showed that AT-MSCs (AT-AT) had a similar morphology in both direct and indirect co-culture where cellular multilayers covered globular deposits (Fig. 4). In contrast, the direct and indirect co-cultures of AT-HU, as well as AT-RAW showed different morphologies. In direct AT-HU, many globular deposits were observed to which cells attached, but in indirect AT-HU only few globular deposits were detectable, which were covered by cellular multilayers. In direct AT-RAW, few cellular accumulations were observed and the culture surface appeared not completely covered with cells. In indirect AT-RAW, cells created very dense cellular multilayers with few globular deposits.

Figure 2. Cell morphology at day 7 (inverted light microscopy). The groups: AT-AT (control), AT-HU, and AT-RAW in direct or indirect culture set-up.

Effect of HUVECs and RAW 264.7 cells on AT-MSC proliferation

Based on results of cellular DNA content, a significantly increased proliferation was observed for direct AT-RAW ($p < 0.001$) during the entire culture period (Fig. 5). The proliferation pattern for direct and indirect AT-HU, as well as for indirect AT-RAW was similar to AT-MSC monoculture, where proliferation increased till day 21 and day 28, after which proliferation decreased to day 35. Remarkably, in these groups (direct/indirect AT-AT, direct/indirect AT-HU, indirect AT-RAW) the proliferation was decreased also at day 14 (results of Donor 1). Table 1 presents an overview of co-culture results from 3 different donors, which shows similar proliferation patterns for all donors. Comparing direct versus indirect co-cultures, an increased proliferation was generally observed for indirect AT-HU (Donor 2 and 3), and direct AT-RAW (all Donors).

Effect of HUVECs and RAW 264.7 cells on AT-MSCs osteogenic differentiation

Early osteogenic differentiation of AT-MSCs was measured

by ALP-activity for both direct and indirect culture set-up (Fig. 6). The results indicated a positive effect of HUVECs on the osteogenic differentiation of AT-MSCs in both direct and indirect culture set-up, where the ALP level was significantly increased during the entire culture period (direct co-culture $\rightarrow p < 0.01$ at day 7, 14, 28; $p < 0.001$ at day 21 and 35; indirect co-culture $\rightarrow p < 0.01$ at day 14; $p < 0.05$ at day 28). RAW 264.7 cells also positively affected AT-MSCs osteogenic differentiation, but not during the entire culture period. A significantly increased ALP-activity was observed for direct AT-RAW at day 28 ($p < 0.001$) and for indirect AT-RAW at day 35 ($p < 0.05$). Next, a decreased osteogenic differentiation was observed during the first three weeks for direct AT-RAW ($p < 0.001 \rightarrow$ day 7; $p < 0.05 \rightarrow$ day 14 and 21). At the same time points, no significant differences were observed for indirect AT-RAW. ALP-activity was similar for the three different donors in direct AT-HU and direct AT-RAW, but varied for indirect AT-HU and indirect AT-RAW (Table 1). For direct versus indirect co-cultures, an increased differentiation was observed for indirect AT-RAW (for all Donors), and direct AT-HU (Donor 2 and 3).

Figure 3. Cell morphology at day 35 (inverted light microscopy). Red arrows indicate mineralization nodules. The groups: AT-AT (control), AT-HU, and AT-RAW in direct or indirect culture set-up.

Effect of HUVECs and RAW 264.7 cells on AT-MSCs mineralization

Ca deposition was measured to monitor the late stage osteogenic differentiation and mineralization of AT-MSCs. Results of Ca deposition were normalized for initial cell seeding number (Fig. 7), which showed that HUVECs positively affected AT-MSCs mineralization in both direct and indirect culture set-up. For direct AT-HU, a significantly increased mineralization was observed at days 14, 21, 28, and 35 ($p < 0.001$). For indirect AT-HU, an increased mineralization was observed at days 21 ($p < 0.05$), 28, and 35 ($p < 0.01$). The mineralization patterns were similar for all three donors in the direct culture set-up, but varied in the indirect culture set-up (Table 1). Direct versus indirect co-cultures showed an increased mineralization for direct AT-HU for all three donors.

RAW 264.7 cells had a positive effect on AT-MSCs mineralization only in an indirect culture set-up (Donor 1), in which a significantly increased mineralization was observed at days 14 ($p < 0.05$), 28, and 35 ($p < 0.01$). For direct AT-RAW, however, mineralization was significantly decreased at days 14, 21, 28, and day 35 ($p < 0.001$). The mineralization pattern of AT-RAW varied among the three donors (Table 3). Direct versus indirect co-culture showed an increased mineralization for all three donors (Table 3).

DISCUSSION

The aim of this study was to evaluate the osteogenic differ-

entiation of AT-MSCs upon co-culture with endothelial cells or macrophages in a direct or indirect culture set-up. Our hypotheses were that 1) endothelial cells and macrophages stimulate AT-MSCs proliferation and osteogenic differentiation, and that 2) these two cell types will more profoundly affect on osteogenic differentiation of AT-MSCs in a direct compared to an indirect culture set-up, because of the possibility for both cell-cell interactions and effects of secreted soluble factors. We observed that HUVECs had a positive effect on osteogenic differentiation and mineralization of AT-MSCs, especially in a direct culture set-up, whereas the same HUVECs had no effect on AT-MSCs proliferation for neither direct nor indirect culture set-ups. Finally, we observed that RAW 264.7 cells had a variable effect on AT-MSCs osteogenic differentiation and mineralization.

An important concept in cell-based bone regenerative strategies is to understand cell-cell interactions between different cell types, which are involved in fracture healing, bone regeneration, remodeling and wound healing processes. This will help to investigate new approaches in bone regenerative research. To understand cellular communication between different cell types, this study used endothelial (HUVECs) and immune cells (RAW 264.7) as co-culture partners for AT-MSCs of three different donors. The results showed that HUVECs have a positive effect on AT-MSC osteogenic differentiation and mineralization, which was consistent for all three donors in direct co-culture set-up. A positive effect of AT-MSC osteogenic differentiation and mineralization was observed also for

Figure 4. Cell morphology at day 35 (scanning electron microscopy). The groups: AT-AT (control), AT-HU, and AT- RAW in direct or indirect culture set-up.

indirect AT-HU, but results were not consistent for all donors. This finding indicates that HUVECs can interact with AT-MSCs and promote osteogenic differentiation via direct and indirect cellular cross-talk [30,31]. Moreover, in direct co-culture set-up, HUVECs more efficiently affected AT-MSCs osteogenic differentiation and mineralization compared to indirect set-up, likely because of simultaneous direct cell-cell contact and actions of secreted soluble molecules.

In contrast, HUVECs showed no effect on AT-MSC proliferation, neither in direct nor in indirect co-culture set-up. However, opposite results have been reported for MSCs isolated from bone tissue, for which HUVECs were able to increase the proliferation, especially in direct co-culture set-up [32]. These results suggest that MSCs from different tissue origins in co-culture with HUVECs may exert different behavior.

RAW 264.7 cells showed a negative effect on AT-MSCs osteogenic differentiation and mineralization, especially in a direct co-culture set-up. Based on morphological evaluation of the co-culture, the number of AT-MSCs appeared very low for direct AT-RAW during the entire culture period. This could

explain the low levels of ALP activity and Ca deposition in the direct AT-RAW group. It is known that RAW 264.7 cells are a type of macrophages, whose main function is to digest any substance recognized as foreign (cellular debris, pathogenic factors such as pathogen bacteria, viruses, cancer cells, etc.) in blood or in tissues [33]. Because AT-MSCs and RAW 264.7 cells derive from different species, AT-MSCs might have been recognized as a foreign cell type and digested by RAW 264.7 cells [34], which would explain survival issues of AT-MSCs in direct co-culture with RAW 264.7 cells. RAW 264.7 cells further are able to differentiate into osteoclasts *in vitro* upon stimulation with RANKL. Osteoclasts are bone resorbing cells and generally in balance with osteoblasts. In direct contact with AT-MSCs, RAW 264.7 cells might show osteoclastic behavior and affect AT-MSCs proliferation and differentiation into osteoblasts [35]. Although in the present study no proof was obtained for osteoclastogenesis of RAW 264.7 cells, the occurrence of osteoclastic differentiation cannot be excluded, particularly in view of the used human PL during cell cultures, which is rich in many signaling molecules and growth factors.

Figure 5. DNA content of AT-MSCs (Donor 1) at day 7, 14, 21, 28, and 35. The groups: AT-AT (control), AT-HU, and AT-RAW in direct or indirect culture set-up. ** $p < 0.01$ and *** $p < 0.001$ indicate the significant differences of AT-HU and AT-RAW compared to AT-AT control, in direct and indirect culture set-up. ## $p < 0.01$ and ### $p < 0.001$ indicate the significant difference between direct and indirect co-culture. AT-MSCs: adipose tissue-derived mesenchymal stromal cells.

For some AT-MSC donors, RAW 264.7 cells showed a positive effect on osteogenic differentiation and mineralization in indirect co-culture set-up. Previously, a positive effect of macrophages on osteogenic differentiation of MSCs was shown by several studies. Few studies indicated that resident tissue macrophages (OsteoMacs) impact bone formation [36,37]. Therefore, it becomes more interesting to investigate how macrophages (from the same species) can affect osteogenic differentiation of AT-MSCs *in vitro*. From all experimental groups, only direct AT-RAW showed an increased proliferation. Based on morphological appearance, however, this was likely a result of the fast growth of RAW 264.7 cells rather than a stimulatory effect of macrophages on AT-MSCs proliferation.

The results of this study contribute to continuing and im-

proving future cell-based bone regenerative therapies, especially related to novel co-culture based strategies. The clinical relevance of this study is that AT-MSCs show high potential for bone tissue regeneration, because AT-MSCs even at half the number of control mono-cultures, in co-culture with other cell types can achieve equal levels of *in vitro* mineralization compared to AT-MSC mono-cultures. Stimulated osteogenic differentiation and mineralization of AT-MSCs was observed especially for co-cultures with endothelial cells, where AT-HU 50–50% ratio showed equal mineralization as AT-MSC mono-culture. The fact that endothelial cells promote AT-MSCs differentiation into osteoblasts makes co-culture system attractive for future preclinical and clinical trials. This effective cellular communication gives an idea about cellular behavior in natu-

Figure 6. ALP activity of AT-MSCs (Donor 1) at day 7, 14, 21, 28, and 35. The groups: AT-AT (control), AT-HU, and AT-RAW in direct or indirect culture set-up. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate the significant differences of AT-HU and AT-RAW compared to AT-AT control, in direct and indirect culture set-up. ## $p < 0.01$ and ### $p < 0.001$ indicate the significant difference between direct and indirect co-culture. AT-MSCs: adipose tissue-derived mesenchymal stromal cells, ALP: alkaline phosphatase, 4NP: p-nitrophenyl phosphate.

Figure 7. Ca content of AT-MSCs (Donor 1) at day 7, 14, 21, 28, and 35. The groups: AT-AT (control), AT-HU, and AT-RAW in direct or indirect culture set-up. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate the significant differences of AT-HU and AT-RAW compared to AT-AT control, in direct and indirect culture set-up. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ indicate the significant difference between direct and indirect co-culture. AT-MSCs: adipose tissue-derived mesenchymal stromal cells.

ral bone micro-environment. However, further research needs to be performed in co-culture systems and a variation of parameters must be investigated to mimic the natural bone micro-environment and entire regeneration cascade. By the help of dedicated scaffold systems, a 3D cellular interaction needs to be analyzed and more cell types need to be included in the same scaffold system. With successful *in vitro* results, *in vivo* research can be designed, where more parameters can be examined in a natural micro-environment.

In conclusion, endothelial cells showed a positive effect on AT-MSC osteogenic differentiation and mineralization. Interestingly, AT-MSCs in AT-HU co-culture set-up, where AT-MSCs were only 50% of total amount, compared to monoculture controls (100% AT-MSCs), achieved equal levels of mineralization. Direct co-culture of AT-MSCs with HUVECs appeared

more effective for osteogenic differentiation and mineralization compared to indirect. This proved our hypothesis that direct co-cultures more profoundly affect osteogenic differentiation and mineralization of AT-MSCs because of direct cell-cell contact. Macrophages affected osteogenic differentiation and mineralization of AT-MSCs variably for indirect co-cultures, whereas direct co-cultures decreased the osteogenic differentiation of AT-MSCs probably related to species differences in the cell types used. The results of this study demonstrate potential for cell combination strategies for bone regenerative therapies.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

The experiments for this article were carried out after institutional approval (IRB no. 2010-00561) and written informed consent.

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