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Short communication

THE PROLIFERATION AND DIFFERENTIATION OF OSTEOBLASTS IN CO-CULTURE WITH HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS: AN IMPROVED ANALYSIS USING FLUORESCENCE-ACTIVATED CELL SORTING

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Abstract: The interaction of osteoblasts and endothelial cells plays a pivotal role in osteogenesis. This interaction has been extensively studied using their direct co-culture *in vitro*. However, co-culture experiments require clear discrimination between the two different cell types in the mixture, but this was rarely achieved. This study is the first to use fluorescence-activated cell sorting (FACS) for the separation and quantitative analysis of the proliferation and differentiation of MG-63 cells grown in direct co-culture with human umbilical vein endothelial cells (HUVECs). The cells of the MG-63 cell line have properties consistent with the characteristics of normal osteoblasts. We labeled HUVECs with fluorescent antibody against CD31 and used FACS to measure the proportions of each cell type and to separate them based on their different fluorescence intensities. The rate of proliferation of the MG-63 cells was estimated based on a count of the total viable cells and the proportion of MG-63 cells in the mixture. The mRNA expression levels of the osteoblast differentiation markers alkaline

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Abbreviations used: ALP – alkaline phosphatise; BSA – bovine serum albumin; Coll-1 – collagen type 1; ECs – endothelial cells; FACS – fluorescence-activated sell sorting; FC – flow cytometry; FCS – fetal calf serum; FITC – fluorescein isothiocyanate; HUVECs – human umbilical vein endothelial cells; mRNA – messenger ribonucleic acid; OBs – osteoblasts; OC – osteocalcin; VEGF – vascular endothelial growth factor

phosphatase (ALP), collagen type 1 (Coll-1) and osteocalcin (OC) in the MG-63 cells were measured via real-time PCR after the separation via FACS. We found that HUVECs stimulated the proliferation of the MG-63 cells after 72 h of co-culture, and inhibited it after 120 h of co-culture. The mRNA expression levels of ALP and Coll-1 significantly increased, whereas that of OC significantly decreased in MG-63 after co-culture with HUVECs. Using FACS for the quantitative analysis of the proliferation and differentiation of osteoblasts directly interacting with endothelial cells could have merit for further co-culture research.

Key words: Osteoblasts, Endothelial cells, Co-culture, Proliferation, Differentiation

INTRODUCTION

Osteogenesis is a complex process that involves the well-orchestrated interaction of various cell types [1, 2]. Close interaction between the bone-forming osteoblasts (OBs) and the vessel-forming endothelial cells (ECs) is crucial for bone vascularization and osteogenesis [3-6]. Several *in vitro* studies investigated the mechanisms underlying this interaction using various co-culture models of these two cell types. Studies using co-culture models without direct contact between OBs and ECs showed that interaction between these cells is mediated by paracrine-acting diffusible factors, namely vascular endothelial growth factor (VEGF), insulin-like growth factor 1, endothelin-1, and bone morphogenetic protein [5, 7, 8]. However, several studies within the last decade indicated that OB differentiation is significantly affected by direct contact with ECs, and cannot be explained exclusively by paracrine mechanisms [9-12]. Therefore, only co-culture with direct contact between OBs and ECs seems to be an adequate model for studying the interaction between these two cell types, because in this model, both direct cell contacts and paracrine mechanisms are considered.

One of the main challenges in experiments with direct co-culture is the necessity to discriminate the phenotype of the various cell types in the cell mixture. This is especially important for the quantitative analysis of both the expression of specific protein markers and the proliferation of each individual cell type. In some previous studies, osteoblasts and ECs were successfully separated via immuno-magnetic separation (IMS) for gene expression analysis [13, 14]. However, magnetic separation does not provide information about the quantitative ratio between osteoblasts and ECs, which is important for estimating the proliferation of the various cell types.

Fluorescence-activated cell sorting (FACS) is a method that allows the quantitative separation of different cell types. This method is a specialized type of flow cytometry. It is widely applied for automatical sorting of heterogeneous cell mixtures depending on light scattering or the fluorescent characteristics of individual cells, and it is usually achieved via the staining of different cell populations with fluorescently labeled antibodies against specific surface

proteins [15, 16]. Recently, FACS was used in a study with the co-culture of OBs and ECs to evaluate the stability of ECs in co-culture [17]. However, to date FACS has not been used to characterize osteoblasts in co-culture with ECs. Therefore, in this study, we investigated the proliferation and differentiation of osteoblast-like MG-63 cells grown in direct co-culture with human umbilical vein endothelial cells (HUVECs) using cell separation by FACS.

MATERIALS AND METHODS

Cell culture

Commercially available osteoblast-like MG-63 cells (American Type Culture Collection, Rockville, USA) and human umbilical vein endothelial cells (HUVECs, Technoclone, Vienna, Austria) were used in this study. The MG-63 cells were cultured in modified Eagle's minimum essential medium (Gibco®, Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 100 U/ml penicillin. The HUVECs were cultured in endothelial cell growth medium (EC medium) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 2 mM L-glutamine, 5 U/ml heparin, 30-50 μg/ml endothelial cell growth supplement (Technoclone, Vienna, Austria) and 20% fetal calf serum (FCS) in 150 cm^2 tissue culture flasks coated with 0.2% gelatine. Both the MG-63 cells and HUVECs were maintained at 37ºC in a humidified atmosphere containing 5% CO_2. All of the experiments were performed using cells between the third and the sixth passages.

Co-culture of MG-63 cells and HUVECs

MG-63 cells and HUVECs were seeded together in each well of a 24-well plate in EC medium at an initial density of 0.5×10^4 and 2.5×10^4 cells/cm^2, respectively. As a control, the MG-63 cells were seeded alone in EC medium at the same density.

Cell proliferation

The proliferation of individual cell types in the co-culture experiment was determined by directly counting the total cell number and measuring the proportions of the individual cell types by flow cytometry in each individual well. The proliferation of the MG-63 cells in the control experiments was determined by direct cell counting.

The total cell number in the co-culture and control wells was counted after 24, 72 and 120 h of culture. Cells were collected by trypsinization. Each well was washed three times with EC medium and examined thoroughly under a microscope to ensure cell removal. Cells from eight different wells were pooled, centrifuged, and resuspended in 200 μl of EC medium. One half of the resulting suspension was used for cell counting, and the other half was used to determine the proportion of each individual cell type. The cells were counted blindly both manually and with an Auto T4 Cellometer Reader (Nexcelom Bioscience LLC, Lawrence, MA). Non-viable cells were excluded by trypan

blue staining. The proportion of MG-63 cells and HUVECs in the co-culture was determined by flow cytometry (FC). The cells were centrifuged and resuspended in 100 µl of ice-cold PBS containing 3% (w/v) bovine serum albumin (BSA) and 0.1% NaN_3 (FC buffer). Afterwards, 20 µl of fluorescein isothiocyanate-conjugated monoclonal anti-human CD31 (eBioscience, San Diego, CA, USA) was added to this cell suspension, which was then incubated on ice in the dark for 30 min. The cells were washed twice after staining, resuspended in 0.5 ml of FC buffer, and the proportions of the different cell types were measured on the flow cytometer (FACS Calibur, Becton Dickenson, CA, USA). Each sample was acquired in triplicate; acquisitions were stopped after 20,000 events. The data was analyzed using the CellQuest 3.3 software (Becton Dickinson, Franklin Lakes, NJ, USA). The CD31-positive cells were considered endothelial cells, and the CD31-negative cells were considered MG-63 cells. The proportion of MG-63 cells in co-culture was calculated for each sample based on the means of three acquisitions.

Gene expression in MG-63 cells sorted by fluorescence-activated cell sorting (FACS)

The expression levels of various specific osteoblast differentiation markers in the MG-63 cells were measured by real-time PCR after 120 h of culture. In the co-culture experiments, MG-63 cells were preliminarily separated from HUVECs by fluorescence-activated cell sorting. The cells from six wells were pooled together, centrifuged and resuspended in 100 µl of FC buffer. The resulting cell suspension was stained with FITC-conjugated anti-human CD31 antibody as in the method described above, and then washed and resuspended in a final volume of 2 ml. The population of MG-63 cells was gated in the CD31-negative cell quadrant, sorted in the exclusion mode, and collected into sorting tubes coated with 4% BSA. The number of sorted events was 50,000 for each sample. In the control experiments, the MG-63 cells grown in a single culture were also passed through a similar staining and sorting procedure.

After collection, the cells were centrifuged, and the total cellular mRNA was isolated and transcribed into cDNA using a TaqMan® Gene Expression Cells-to-CT™ kit (Ambion/Applied Biosystems, CA, USA) according to the manufacturer's instruction. Real-time PCR was performed on an Applied Biosystems Step One Plus real-time PCR system (Applied Biosystem, CA, USA) using the Taqman® gene expression assays with the following ID numbers (all from Applied Biosystems, CA, USA): alkaline phosphatase, Hs01029144_m1; osteocalcin, Hs00609452_g1; Collagen type I, Hs00164004_m1; Runx-2, ref. Hs00231692_m1; β-actin, Hs99999903_m1. Triplicate PCR reactions were prepared for each sample under the following thermocycling conditions: initiation at 95ºC for 10 min, then 40 cycles each consisting of denaturation at 95ºC for 15 s and hybridization-elongation at 60ºC for 1 min. The point at which the PCR product was first detected above a fixed threshold (termed the cycle threshold, C_t), was determined for each sample. Changes in the expression of the

target gene were calculated using $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_t^{target}-C_t^{\beta\text{-actin}})_{sample}-(C_t^{target}-C_t^{\beta\text{-actin}})_{control}$, taking samples of MG-63 cells grown in single culture as the control. The real-time PCR experiments were repeated three times.

Quantitative measurement of alkaline phosphatase activity

The activity of alkaline phosphatase (ALP) was measured similarly to the method described in our previous studies [18] after 120 h of culture. Cells were divided into two groups: one was used to determine the total number of MG-63 cells by direct cell counting and flow cytometry as described above, whereas the other was lysated in 200 μl PBS containing 0.2 % Triton X-100 and homogenized by sonification. The ALP activity was assayed using the conversion of a colorless *p*-nitrophenyl phosphate to a colored *p*-nitrophenol according to the manufacturer's protocol (Sigma, St. Louis, MO, USA). The color changes were measured spectrophotometrically at 405 nm. The amounts of resulting *p*-nitrophenol released by the cells were quantified by comparison with a standard curve and normalized to the total number of MG-63 cells at the end of the experiment. ALP activity experiments were repeated three times. The data is expressed as ratios of nanomoles of inorganic phosphate (Pi) cleaved by the enzyme in 30 min per 100,000 cells.

Statistical analysis

All the statistical analyses were performed using the statistic program SPSS 14.0. The data is expressed as the means ± SD. The normal distribution of all of the data was tested with the Kolmogorov-Smirnov test. After confirming the normal distribution, the statistical differences between the co-culture and single culture groups were analysed using the *t*-test. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Cell proliferation

Fig. 1 shows changes in the proportions of MG-63 cells and HUVECs in the co-culture experiments. As can be seen, over time the proportion of MG-63 in co-culture gradually increased, whereas that of HUVECs gradually decreased. The ratio of MG-63 and HUVECs changed from 1:5 at the beginning of the experiments to about 1:1 on the 5^{th} day (Fig. 1B).

Fig. 2 shows the proliferation of MG-63 cells in the co-culture with HUVECs as well as in the control single-culture experiment. Within the observed period, the number of MG-63 cells increased continuously in both the co-culture and control experiments. After 72 h, the cell number of MG-63 cells in the co-culture group was significantly higher than that in the control group ($P < 0.01$). By contrast, after 120 h of culture, the cell number of MG-63 cells in the co-culture group had become significantly lower than that in the control group ($P < 0.01$).

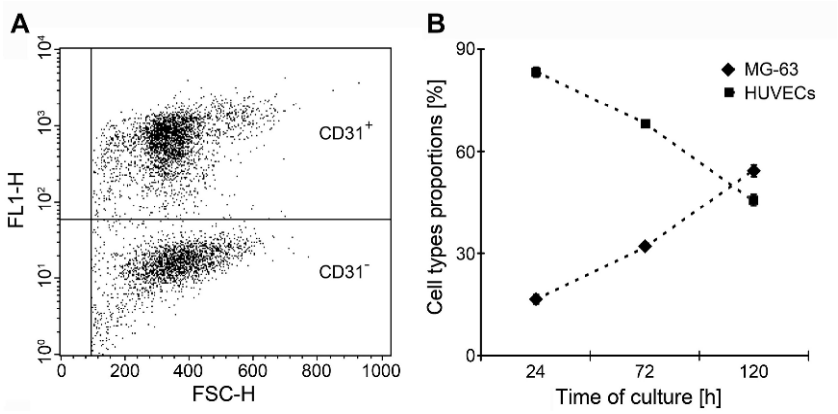

Fig. 1. Changes in the proportions of MG-63 cells and HUVECs in the co-culture experiments. MG-63 cells and HUVECs were seeded at an initial ratio of 1:5. The proportions of the individual cell types at the various time points were measured by FACS. A – The original dot-plot obtained via FACS analysis of the cell suspension stained with FITC-conjugated anti-human CD31 antibody. Two cell populations could clearly be distinguished: the CD31-positive cells ($CD31^+$, upper quadrant) were identified as HUVECs, and the CD31-negative cells ($CD31^-$, lower quadrant) were identified as MG-63 cells. The proportions of the different cell types in suspension were determined based on the cell number in each population. B – Changes in the proportions of MG-63 cells and HUVECs during the co-culture experiments. The data is shown as the means ± SD of one representative experiment.

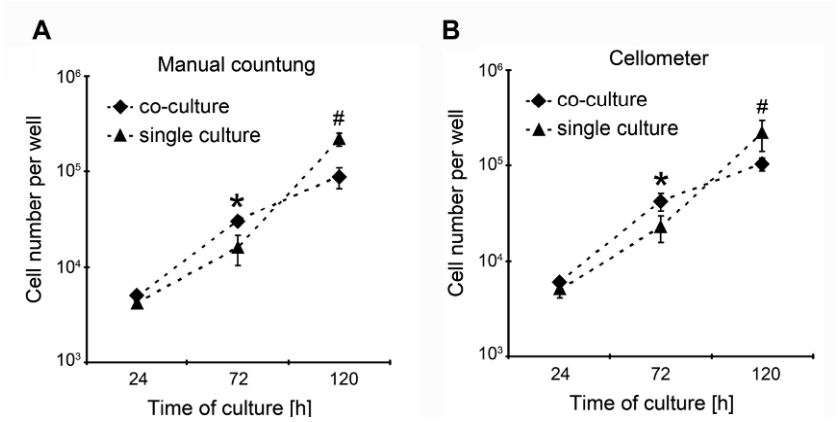

Fig. 2. The proliferation of MG-63 cells in co-culture with HUVECs and in the control experiments. The cell numbers of MG-63 cells in the co-culture and control experiments were determined based on manual (A) and automatic (B) cell counting. The number of MG-63 cells in the co-culture was calculated by multiplying the total cell number by the percentage of MG-63 cells determined by FACS. The data is shown as the means ± SD of three independent experiments. * means a significantly higher ($P < 0.01$) number of MG-63 cells in the co-culture experiments compared to the control experiments. # means a significantly lower ($P < 0.01$) number of MG-63 cells in the co-culture experiments compared to the control experiments.

The gene expression level of various osteogenic factors in MG-63 cells

Fig. 3 shows the changes in the gene expression levels of various osteogenic factors in MG-63 upon co-culture in direct contact with HUVECs. We found that the mRNA expression levels of ALP and collagen type I significantly increased whereas that of osteocalcin significantly decreased in MG-63 cells cultured in direct contact with HUVECs, compared to the control cells. No significant differences were found in the mRNA level of Runx-2 in MG-63 upon co-culture with HUVECs.

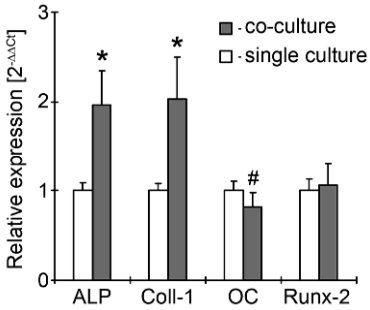

Fig. 3. Changes in the mRNA expression levels of osteogenic differentiation markers in MG-63 cells after co-culture with HUVECs. The mRNA expression level of alkaline phosphatase, collagen type I, osteocalcin, and runx-2 were analyzed via quantitative real-time PCR. β-actin was taken as an endogenous house-keeping gene. Total mRNA was isolated from MG-63 cells in both co-culture and single culture groups after 120 h. MG-63 cells were separated from HUVECs using the FACS method. The data is presented as the relative amount of mRNA with the formula $2^{(-\Delta\Delta Ct)}$ taking MG-63 cells in single culture as a control. * means significantly higher compared to MG-63 cells in single culture ($P < 0.01$). # means significantly lower compared to MG-63 cells in single culture ($P < 0.05$).

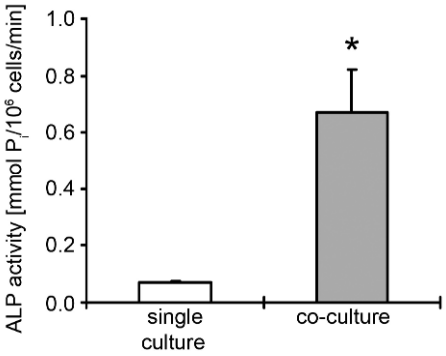

Fig. 4. Changes in the alkaline phosphatase activity of MG-63 cells upon co-culture with HUVECs. ALP activity was determined after 120 h of culture by measuring the release of *p*-nitrophenol from *p*-nitrophenolphosphate. The results are expressed in mmol of Pi cleaved by 10^5 MG-63 cells per min. The data is shown as the means ± SD of three independent experiments. * means significantly higher than in single culture ($P < 0.05$).

Alkaline phosphatase activity of MG-63 cells

Fig. 4 shows the alkaline phosphatase activity of MG-63 cells in the co-culture and control experiments. As can be seen, the alkaline phosphatase activity of MG-63 cells grown in direct contact with HUVECs was about 10 times higher ($P < 0.05$) than that of MG-63 alone (Fig. 4).

DISCUSSION

In this study, we used osteoblast-like MG-63 cells, which are often used as a model of osteoblasts, especially in studies testing different biomaterials. MG-63 cells are derived from malignant bone tumor and share some osteoblastic features. In particular, these cells express alkaline phosphatase, osteocalcin and collagen type I [18-21]. However, some studies have indicated several differences between MG-63 cells and primary osteoblasts in the gene expression profile and expression of various receptors [19, 22]. Despite these differences, we used MG-63 cells in this study, mainly because they represent a cell line with constant properties, whereas the characteristics of primary osteoblasts may differ depending on the source, donor, and isolation method [23-25]. However, care should be taken when extrapolating data obtained with osteoblast-like MG-63 cells to the situation *in vivo*.

Direct contact between OBs and ECs seems to be a crucial factor that substantially influences the osteogenic differentiation of OBs. Therefore, to study the effect of ECs on OB proliferation and differentiation, it is very important to find co-culture conditions under which a sufficient amount of ECs is present during the whole experiment. One previous study showed that in co-culture experiments, the proliferation rate of ECs is lower than that of OBs [17]. The proportion of ECs dropped from an initial level of 40% to about 10% after one week in experiments using both MG-63 cells and primary oteoblasts. Such a low proportion of ECs would result in a situation where some osteoblast cells have no direct contact with ECs. In this study, we seeded MG-63 and HUVECs at an initial ratio of 1:5, and used a specific EC medium for all of the co-culture experiments. We found that under these conditions, a high proportion of ECs persists in co-culture even after 120 h, which allows a continuous interaction of OBs with ECs. Our observation is in line with the results of a previous study of co-culture of MG-63 cells with human dermal microvascular endothelial cells (HDMEC) [26]. When the HDMEC:MG-63 were seeded at initial ratios of 5:1 or 10:1, a sufficient amount of both cell types was present after one week's culture, whereas at initial ratios of 1:1, 1:5 and 1:10, no endothelial cells were present.

Traditional methods for estimating the rate of cell proliferation are based on metabolic assays and/or DNA synthesis quantification [27], but they cannot be appropriately used in experiments with direct co-culture because of the variability of the measured parameters between the different cell types [28]. In order to overcome these difficulties, we determined the total viable cell number

by direct counting, and measured the proportions of each individual cell type via flow cytometry. The combination of these methods yielded information about the proliferation of each individual cell type. We found that HUVECs stimulate the proliferation of MG-63 cells within 72 h of co-culture and inhibit it within 120 h of co-culture. The mechanisms underlying these effects are not clear. A previous study suggested that osteoblasts may stimulate the proliferation of ECs by producing VEGF, and that the stimulated ECs may reciprocally enhance the proliferation of osteoblasts in direct co-culture [5]. The inhibition of MG-63 cell proliferation by HUVECs after 120 h of culture could be due to the mechanisms recently described by Finkenzeller *et al.* [29]. According to this direct co-culture study, HUVECs inhibited proliferation of primary OBs due to down-regulation of platelet-derived growth factor receptor expression on the OB surface. Summarizing, our observation implies that the interaction of osteoblasts with ECs might have a complex nature, resulting in the stimulation of OB proliferation at earlier stages, and its inhibition at later stages.

The next aim of our study was to investigate how HUVECs influence the osteogenic differentiation of MG-63 cells. In this study, we used real-time PCR to investigate the mRNA expression levels of the early osteoblast differentiation markers ALP and Coll-1 [30-32], the late osteoblast differentiation marker OC [33], and the major OC regulator Runx-2 [34]. Before the real-time PCR experiments, the MG-63 cells were separated from the HUVECs by FACS. Such a separation was definitely required, because to quantitatively analyze the mRNA levels of the target genes, one needs to relate them to β-actin, which is present in both MG-63 and HUVECs. Furthermore, we measured the alkaline phosphatase activity of the cells in relation to the number of MG-63 cells, which was possible because HUVECs exhibited no ALP activity (data not shown).

Our data showed that HUVECs differently affect the expression of early and late differentiation markers in MG-63 cells. In particular, the mRNA expression levels of ALP and Coll-1 and the ALP activity were significantly higher in MG-63 cells grown in direct contact with HUVECs than in the control cells. By contrast, MG-63 cells grown in co-culture with HUVECs exhibited significantly lower OC expression and a similar level of Runx-2 expression compared to the control cells. Unfortunately, there is no previous data on the expression of osteogenic markers in MG-63 cells grown in co-culture with EC. However, the results of our study are generally in agreement with previous data obtained in studies with direct co-culture of primary osteoblasts and ECs for alkaline phosphatase [5, 10-12, 14] and collagen type I [10, 12]. The effect of ECs on the expression level of OC was controversially discussed in previous studies. Villars *et al.* showed that OC synthesis by human bone marrow stromal cells (HBMSCs) decreased in co-culture with HUVECs [11]. Guillotin *et al*. showed that the mRNA level of OC and Runx-2 in human osteoprogenitors from HBMSCs was down-regulated after 48 h of direct co-culture with HUVECs [14]. Our findings support the view that endothelial cells inhibit OC expression in osteoblasts. By contrast, two other studies reported significantly enhanced OC production by

HBMSCs in co-culture with endothelial cells [13, 35], which appears to contradict our findings. However, these studies used ELISA and measured the OC produced by HBMSCs and accumulated during a certain time, whereas we measured the expression level at a fixed time point. The effects of inhibiting OC expression in our co-culture experiments with MG-63 osteoblast-like cells and in previous studies with HBMSCs [11, 14] suggest that endothelial cells inhibit osteogenesis. For the situation *in vivo*, such inhibition was suggested to be an important mechanism preventing mineral deposition within the vessels [36].

Another important aspect of our study is that the initial cell number of MG-63 cells and HUVECs in the co-culture experiments was 3×10^4 cells/well, whereas in the control single-culture experiments, the initial cell number of MG-63 cells was 0.5×10^4 cells/well. It is well known that a higher cell density generally inhibits cell proliferation and promotes cell differentiation. However, this fact seems not to influence the conclusions of our study. After 72 h, the proliferation of MG-63 cells in the co-culture experiments was higher than that in the single culture despite the higher cell density. After 120 h, the total cell number of MG-63 cells in the single culture was about twice as high as that in the co-culture. However, since the ratio of MG-63 cells and HUVECs in the co-culture experiments was about 1:1, one can conclude that the cell density in the co-culture experiments was similar to that in the single culture experiments. Thus, the inhibition of MG-63 cell proliferation after 120 h in the co-culture experiments seems not to be due to different cell densities. Similarly, the expression of specific markers was measured after 120 h of culture, and therefore, we assume that changes in the gene expression are not due to different cell densities. However, the possibility that different cell densities within the time course of the experiment influence the results cannot totally be excluded.

In conclusion, in this study, we used FACS for the quantitative analysis of the proliferation and differentiation of osteoblasts grown in direct co-culture with ECs. We found that HUVECs have complex effects on MG-63 cells. HUVECs stimulated the proliferation of MG-63 cells at the early stages and inhibited it at the late stages of co-culture. In addition, HUVECs stimulated the expression of early osteogenic markers in MG-63 cells but inhibited that of the late osteogenic marker. Fluorescence-activated cell sorting seems to be an important approach that allows cell separation, and quantitative analysis of cell proliferation and differentiation has potential merits for application in direct co-culture research.

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