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Microwell Geometry Modulates Interleukin-6 Secretion in Human Mesenchymal Stem Cells

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ABSTRACT

The therapeutic effect of mesenchymal stem cells (MSCs) has been investigated in various clinical applications, in which their functional benefits are mainly attributed to the secretion of soluble factors. The enhancement of their therapeutic potential by physical and chemical properties of cell culture substrate is a safe and effective strategy, since they are highly sensitive to their microenvironment such as the elasticity and surface topography. In this study, we demonstrated that the geometry of polymeric substrate regulated the interleukin-6 (IL-6) secretion of human adipose derived MSCs. Polystyrene substrates comprising arrays of square-shaped (S50) or round-shaped (R50) microwells (side length or diameter of 50 µm and depth of 10 µm) were prepared by injection molding. Cellular apoptototic rate of MSCs was not affected by the microwell geometry, while the upregulated secretion of IL-6 and the enhancement of nuclear transcription factor STAT3 were detected in MSCs seeded on S50 substrate. The geometry-dependent modulatory effect was highly associated with ROCK signaling cascade. The inhibition of ROCK abolished the disparity in IL-6 secretion. These findings highlight the possibility to steer the secretion profile of stem cells via microwell geometry in combination with the manipulation of ROCK signaling pathway.

INTRODUCTION

Mesenchymal stem cells (MSCs) have shown several advantages in regenerative medicine including the abundance, homing ability, multi-linage differentiation capacity and immunoregulatory properties. They contribute to regenerative process via not only direct differentiation into specific cell types but also secretion of paracrine signaling molecules [1-3]. Thus, regulation of stem cell secretome has emerged as a promising cell-free strategy in

regenerative therapy [4]. Improving therapeutic potential of MSCs by fine-tuning the structure and composition of biomaterials has become a safe and effective strategy, as MSCs are highly sensitive to the local physical properties of microenvironment such as the elasticity [5] and surface topographic features [6-8]. Interleukin-6 (IL-6), as a major pro-/anti-inflammatory, pro-/anti-apoptotic and pro-angiogenic cytokine, plays a central role in neuroprotection [2], maintaining stemness of MSCs [9], as well as tissue regeneration [1]. It has been shown that endogenous IL-6 secretion from stem cells can trigger the activation of its downstream factor STAT3, which was indispensable for liver regeneration following hepatectomy [10]. In the present study, to assess the potential capability of steering MSC secretome via surface microstructures and determine the underlying molecular mechanism that mediates the signal transduction process, human adipose derived MSCs were seeded in microstructured polystyrene (PS) inserts compressing arrays of square-shaped (S50) or round-shaped (R50) microwells. The regulatory effect of microstructured surfaces on cellular apoptosis, STAT3 expression, IL-6 secretion of MSCs and the involved ROCK signaling pathway were investigated.

EXPERIMENT

The characterization of microstructured PS substrates with S50 or R50 microwells and the vitronectin adsorption and distribution were described in the previous study [7]. MSCs were isolated from human adipose tissue obtained by abdominal liposuction from a female donor after informed consent (No.: EA2/127/07; Ethics Committee of the Charité - Universitätsmedizin Berlin). Cells were cultured in DMEM medium containing 10 vol% fetal bovine serum and were seeded as a single cell suspension on the microstructured substrates with a density of 1×10^4 cells/cm^2. At day 10, Annexin V-PE and 7-AAD were counterstained and detected by flow cytometer for calculating cell apoptotic rate. Expression level of STAT3 and GAPDH were detected by specific labeling with mouse anti-human STAT3 (1:1000 in blocking buffer) and rabbit anti-human GAPDH antibodies (1:1000 in blocking buffer). Human IL-6 DuoSet enzyme-Linked immunosorbent assay (ELISA) kit was used for quantification of IL-6 secretion level in conditioned medium. 10 μM of Y-27632 was used as a selective ROCK signaling pathway inhibitor for IL-6 secretion analysis. Statistical analysis was performed using the two-tailed

independent-samples t test, and a p value < 0.05 was considered to be statistically significant. Data are presented as mean ± standard deviation if not indicated otherwise.

DISCUSSION

In this study, we hypothesized that the IL-6/STAT3 signaling and IL-6 secretion of MSCs could be effectively regulated by microwells. The key elements for monitoring the regulatory effect of microstructured surfaces on MSC secretome was shown in figure 1. MSCs were seeded into the inserts and cultured for 10 days. As observed in our previous study [7], MSCs could attach and migrate freely on both microstructured substrates, while the cells preferred to migrate into the microwells as early as 2 hours after seeding (1-2 cells/microwell). The cellular apoptosis rate and the expression of nuclear transcription factor STAT3 were assessed. In order to evaluate the amount of IL-6 secretion by MSCs, conditioned media were collected at different time points and the IL-6 was quantified using ELISA. The function of ROCK, the master cytoskeleton regulator, was particularly investigated via an inhibition assay.

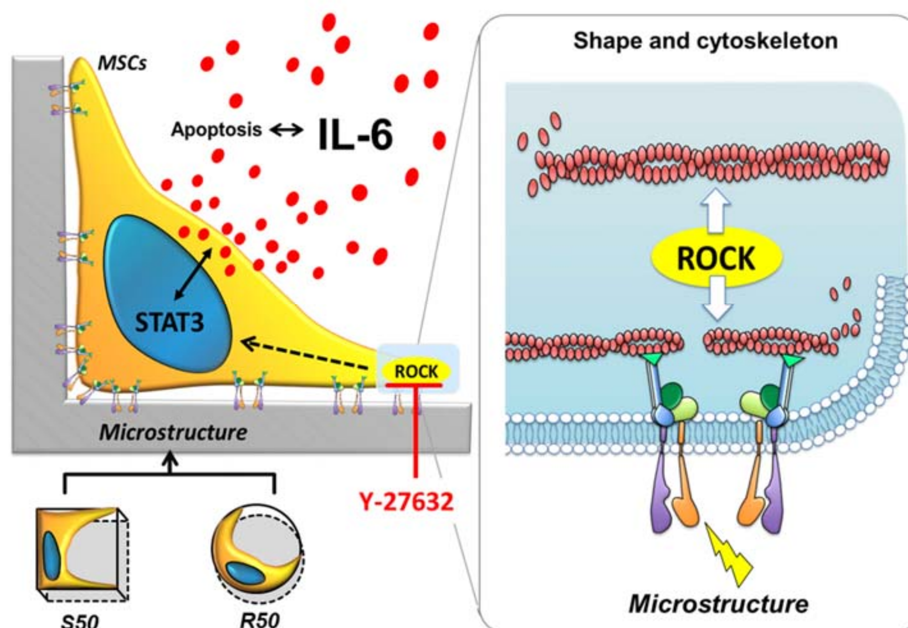

Figure 1. Schematic illustration of the hypothesis and the key elements for investigating the regulatory effect of microwell geometry on human adipose derived MSCs and the potentially involved ROCK and STAT3 signaling transduction pathway for steering IL-6 secretion.

Microwell geometry does not affect cellular apoptosis of MSCs

Production and secretion of endogenous IL-6 are dependent to the cellular apoptosis of MSC [11]. Moreover, it has been reported that the apoptotic rate can be influenced by the geometry and adhesive area of micropatterns. However, strong apoptosis was only observed in cells on the islands with a size less than 75 μm^2, but not on the larger islands [12]. Here, we used microwells with an area much larger than the reported size threshold regarding apoptosis induction. In consistence with the reported observation, we found that the rate of apoptotic cells, which defined as Annexin V-PE^+ 7-AAD^- cells (Figure 2A) was not affected by the geometric cues of microwells after 10 days of cultivation according to the flow cytometry analysis (Figure 2B).

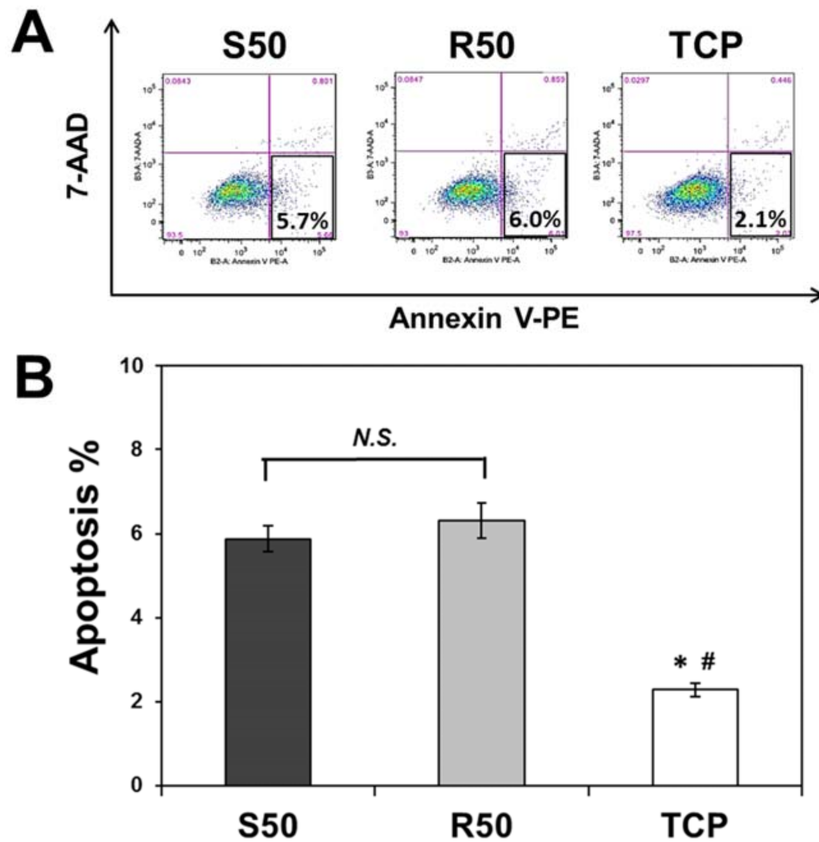

Figure 2. Cellular apoptosis of MSCs. Representative dot plots of Annexin V-PE$^+$ (labeling both of apoptotic and necrotic cells) 7-AAD$^-$ (positive for necrotic cells)apoptotic cell populations (A) and quantitative analysis of apoptotic rates of cells growing on S50, R50 and TCP surfaces (B). The smooth tissue culture plate (TCP) was used as control. (*$p<0.05$ vs S50, #$p<0.05$ vs. R50, n=3).

Microwell geometry alters canonical IL-6/STAT3 signaling

It has been established that cytokine IL-6 in the secretome of MSCs can activate the STAT3 signaling pathway through accumulating both phosphorylated [10] and non-phosphorylated STAT3 [13]. Moreover, as one of the key stemness marker, STAT3 in cancer stem cells could be influenced by the geometry of adhesive island which resulted in the change of cell shape [14]. Notably, in the present study, the thick bands of STAT3 in MSCs growing on S50 for 10 days were observed (Figure 3A) and the significant upregulation of STAT3 overall expression level

(normalized with housekeeping protein GAPDH) in response to the geometry of S50 microwells was further demonstrated by quantitative analysis (Figure 3B).

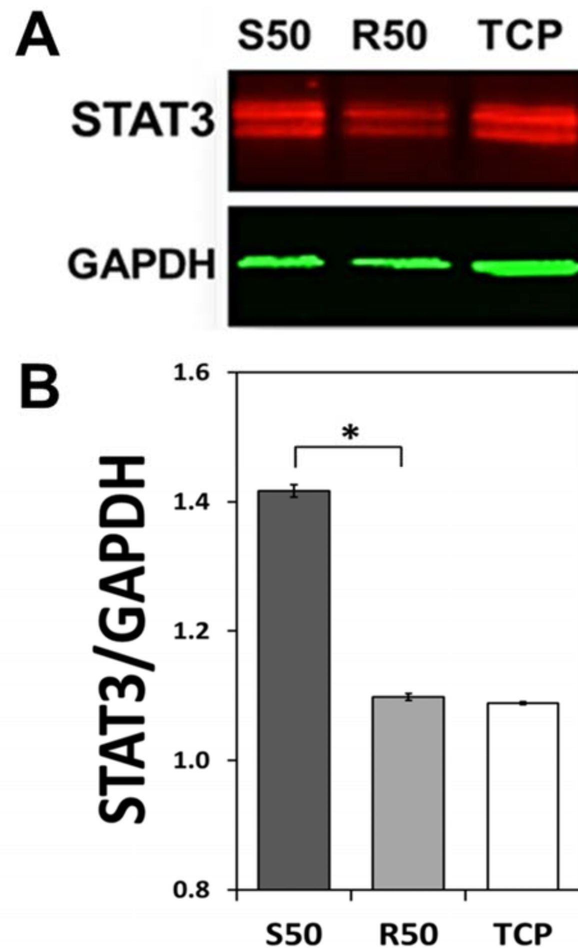

Figure 3. Intracellular STAT3 expression in MSCs. (A) Representative western blotting bands for STAT3 and housekeeping protein GAPDH in total proteins that extract from MSCs growing on microstructured and smooth TCP surfaces. (B) The protein expression level of total STAT3 by MSCs cultured on S50 was higher compared to that on R50 substrates at day 10, as quantified based on western blotting images. GAPDH was used as total protein loading control for normalization. (*$p<0.05$, $n=3$)

Geometry modulates IL-6 secretion via ROCK pathway

Microscale features including geometry have been reported to be involved in modulating MSC differentiation [6, 15]. However, to our knowledge, there is still lack of investigations focusing on the secretome alteration of MSCs by simply using geometric microstructures. Here, the level of total STAT3 was increased in MSCs growing on S50 substrate after 3 days culture (Figure 3B). To further examine and quantify the secretion of IL-6, freshly collected conditioned media from MSCs on S50, R50 and smooth TCP surfaces were analyzed via ELISA. The microstructured substrates were designed to accommodate one single cell in one microwell, with an optimal inter-microwell space for cell spreading to exclude the effect of multiple microwells on a single cell [7]. Approximately 14.5% increase of IL-6 secretion was found in MSCs on S50 over R50 after 24 hours (Figure 4). We speculate that the regulatory effect of the microwells on MSCs would be more pronounced by further increasing the structured area, given the lower percentage of microwell-covered area to the total area in the present study. In our previous study, we have demonstrated that the microwells could effectively regulate MSC adhesion and F-actin organization [7]. In addition, we showed S50 microwells strongly upregulated ROCK signalling than R50 [7]. It has been well documented that ROCK protein, as an active effector of Rho A, influences cell behavior and function mainly through its regulation in actin-cytoskeleton assembly and cell-substrate interaction [16, 17]. Moreover, ROCK could activate the feedback loop between IL-6 and STAT3, resulting in the activation of STAT3 and IL-6 secretion [13, 18, 19]. In present study, an upregulated STAT3 and IL-6 expression was observed in cells on S50 substrate which was in consistence with the previous studies. Taken together, we speculate that the elevated IL-6 in cells on S50 was via the following mechanism: 1) the geometry of S50 microwell could regulate cell adhesion and F-actin cytoskeleton, which promoted the activation of ROCK signaling; 2) the feedback loop of IL-6/STAT3/IL-6 could be then activated upon upregulated ROCK, and IL-6 secretion was consequently increased. To study the function of ROCK in IL-6 secretion, the selective inhibitor Y-27632 was applied to MSCs 12 hours after cell seeding. The inhibition of ROCK markedly abolished the difference of IL-6 secretion between the cells on different surfaces (Figure 4). Interestingly, the IL-6 secretion level of MSCs on S50 substrate was higher than that in MSCs on TCP. It suggested that square-shaped microstructure might be a preferable surface feature for biomaterials used in IL-6 participated regenerative applications.

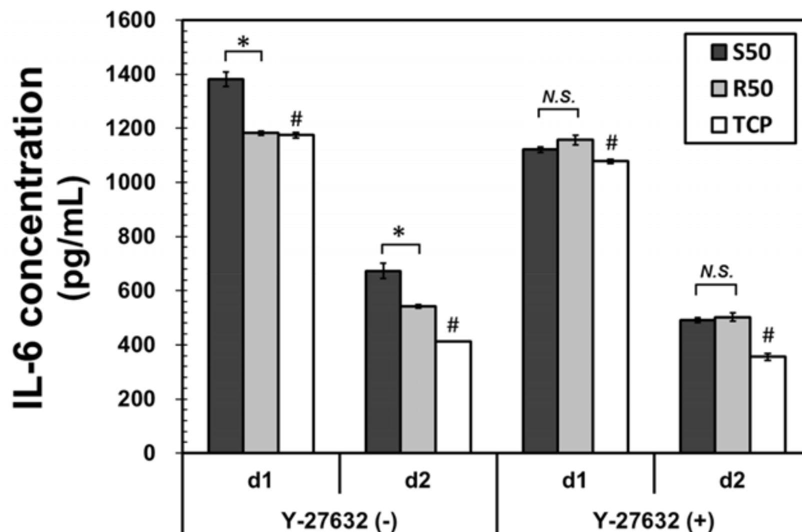

Figure 4. Influence of ROCK signaling on IL-6 secretion. Conditioned media of cells culture were harvested at different time points. The concentration of IL-6 was detected via ELISA and normalized by cell number. The elevated IL-6 secretion on S50 was diminished when ROCK was blocked by Y-27632 (*p<0.05, #p<0.05 vs. S50, n=3).

CONCLUSIONS

In this study, we demonstrated that the secretion of human MSCs could be modulated using microwells in combination with manipulating ROCK signaling pathway, without induction of cellular apoptosis. The elevated secretion of IL-6 and its associated nuclear transcription factor STAT3 was detected in MSCs growing on S50 substrate. The microwell geometry-dependent modulatory effect was highly regulated by ROCK signaling. The inhibition of ROCK eliminated the differences in IL-6 secretion between cells on different substrates. These findings highlight the possibility to modulate and control the stem cells via microwell geometry in combination with the manipulation of related signaling pathways, and therefore provide the valuable information for

designing and developing medical implants or cell culture materials to promote the tissue regeneration.

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