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Modulation of the mesenchymal stem cell migration capacity via preconditioning with topographic microstructure

Zhengdong Li[1, 2], Xun Xu[1, 2], Weiwei Wang[1], Karl Kratz[1, 4], Xianlei Sun[1, 3], Jie Zou[1, 2], Zijun Deng[1,2], Friedrich Jung[1, 4], Manfred Gossen[1], Nan Ma[1, 2, 4, *] and Andreas Lendlein[1, 2, 3, 4, *]

[1] Institute of Biomaterial Science and Berlin-Brandenburg Center for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Teltow, Germany

[2] Institute of Chemistry and Biochemistry, Freie Universität Berlin, Berlin, Germany

[3] Institute Biochemistry and Biology, Universität Potsdam, Potsdam, Germany

[4] Helmholtz Virtual Institute "Multifunctional Biomaterials in Medicine", Teltow, Germany

* To whom correspondence should be addressed:

Prof. Dr. Nan Ma, Prof. Dr. Andreas Lendlein, Institute of Biomaterial Science, Helmholtz-Zentrum Geesthacht, Kantstr. 55, 14513 Teltow.

Email: nan.ma@hzg.de, andreas.lendlein@hzg.de

Phone: +49 (0)3328 352-235

Fax: +49 (0)3328 352-452

Abstract

Controlling mesenchymal stem cells (MSCs) behavior is necessary to fully exploit their therapeutic potential. Various approaches are employed to effectively influence the migration capacity of MSCs. Here, topographic microstructures with different microscale roughness were created on polystyrene (PS) culture vessel surfaces as a feasible physical preconditioning strategy to modulate MSC migration. By analyzing trajectories of cells migrating after reseeding, we demonstrated that the mobilization velocity of human adipose derived mesenchymal stem cells (hADSCs) could be promoted by and persisted brief preconditioning with the appropriate microtopography. Moreover, the elevated activation levels of focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK) in hADSCs were also observed during and after the preconditioning process. These findings underline the potential enhancement of *in vivo* therapeutic efficacy in regenerative medicine via transplantation of topographic microstructure preconditioned stem cells.

Key words: Mesenchymal stem cells, Precondition, Microstructure, Migration, FAK-MAPK

1 Introduction

Mesenchymal stem cells (MSCs) have been considered as a promising cell source for cell transplantation and tissue engineering [1-3]. The improvement of MSC migration capacity *in vivo* towards the damaged tissue such as infarcted myocardium is one of the major challenges in stem cell therapy [4-7]. An *in vivo* study with a rat myocardial infarction model revealed that merely 1% of MSCs injected systemically can reach the ischemic myocardial area [8]. Further, since MSCs did not preserve a long lifespan after transplantation [9,10], the beneficial effects from transplanted MSCs may mainly be attributed to the rapid targeting to the site of injury. Thus, cell migration to the lesion site remains the lingering problem in MSC-based therapies and it would be of great benefit to enhance the MSCs migration capacity prior to their transplantation.

Several biochemical approaches have been developed to promote MSC migration. For instance, short-term exposure of MSCs to hypoxia and pretreating MSCs with cytokines and growth factors such as IL-6 and HGF could upregulate the expression of chemokine receptors and therefore promote the MSC migration [11-13]. Besides, overexpression of chemokine receptors such as

CXCR4 on MSC surfaces via genetic engineering can also efficiently enhance the migration capacity and result in an improved recovery after myocardial infarction [14]. However, genetically modified MSCs may not yet be feasible for therapeutic applications in terms of safety issues. Therefore, preconditioning of MSCs with biochemical factors is currently widely applied in regulating cell migration capacity.

It is particularly attractive to induce and modulate MSC migration without introducing artificial biochemical factors, through physical, especially mechanical properties, of biomaterials. From this point of view, modification of wettability [15] and rigidity [16,17] of materials has been reported to influence the cell motility. Moreover, it was demonstrated that cells could reach their maximum migration capacity when treated with optimal mechanical cues [18-21]. A material surface with nanoscale roughness has been described as a strong modulator in regulating cell migration [22,23]. Materials with microscale surface roughness were able to regulate cell adhesion, proliferation, and differentiation [24,25]. One up-to-date study indicated that the proper level of microscale roughness could alter osteoblast migration speed [26]. Meanwhile, polymeric material with the advantage of tailorable physical and mechanical properties provides a broad platform to study cell-material interactions. Strategies such as surface structure manipulation [27] and protein patterning [28] on polymeric materials were demonstrated to induce specific effects on cells. Thus, it is of high interest to study the influence of polymeric materials with surface microscale structures on the migration capacity of MSCs.

Focusing on the interface between cell and material surface, the cell transmembrane receptor integrin, is responsible for sensing and translating external mechanical signals into cellular biochemical information [29,30]. Intracellularly, integrin interacts with focal adhesion kinase (FAK) via the cytoskeletal protein talin [31]. FAK has been demonstrated to play a key role in cell migration [32,33]. Furthermore, FAK activation is connected to the mitogen-activated protein kinase (MAPK) cascade [34]. Thus, it is of great importance to study the effects of topographic microstructures on stem cell migration capacity and the activity of FAK and MAPK, which may reveal the underlying mechanism and present a safe and robust approach to increase the therapeutic efficacy of MSCs in regenerative medicine. Here, we hypothesize that microscale surface roughness can influence the stem cell migration capacity through FAK and MAPK activity.

To test our hypothesis, polystyrene (PS) cell culture inserts with topographic microstructures defined in three distinct roughness levels (R0, R1 and R2) on the bottom surfaces were fabricated to explore the effects of microstructures on the migration capacity of human adipose-derived mesenchymal stem cells (hADSCs). The morphology and focal adhesion (FA) of hADSCs were observed. The influence of microstructures on migration capacity was evaluated *in vitro* using time-lapse and gap closure assays as schematically illustrated in Fig. 1. Furthermore, the activation of FAK and MAPK in response to topographic microstructures was extensively investigated.

2 Materials and methods

2.1 Cell culture surfaces

As described before [35], polystyrene inserts with different microstructures on the bottom were manufactured by injection moulding. The prepared inserts were sterilized using gas sterilization. The roughness level of the insert bottom with different microstructures was determined as described previously [36]. In brief, optical profilometer (MicoProf 200, FRT - Fries Research & Technologie GmbH, Bergisch Gladbach, Germany) equipped with a CWL 300 μm chromatic white-light sensor was used to measure the level of root mean square roughness (*R*q). The data were acquired with the software AQUIRE (ver. 1.21) and were evaluated with the software MARK III (ver. 3.9). To enhance the cell adhesion, the bottom surfaces of the prepared inserts were coated with 300 μl of human fibronectin solution (10μg/ml in PBS, Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 ºC for 1 h.

2.2 Cell cultivation

hADSCs isolated from human adipose tissue expressed the phenotypic markers CD90, CD105 and CD73 [37]. The adipose tissue was obtained by abdominal liposuction from a female donor after informed consent (No.: EA2/127/07; Ethics Committee of the Charité - Universitätsmedizin Berlin, approval from 17.10.2008). The isolated hADSCs were cultured in human adipose-derived stem cell medium (ADSC^{TM} growth medium, Lonza, Walkersville, MD, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3 Migration assay

2.3.1 Gap closure assay

A 24-well wound healing assay plate (CytoSelect™ 24-Well wound healing assay plate, Cell Biolabs. INC, San Diego, USA) was used to assess cell migration according to manufacturer's instructions. In brief, hADSCs cultured on surfaces with different microstructures for 2 days to 14 days were collected and then reseeded in a 24-well wound healing assay plate at a density of 3×10^4 cells per cm^2. After overnight adhesion, a gap was created by removing the baffle in the center of the well, and images of the gaps at each time point were taken with a digital camera connected to a phase-contrast Olympus microscope (IX81 motorized inverted microscope, Olympus, Hamburg, Germany, ×10 objective). The same visual field was marked and used throughout the experiment. The gap area was measured by Image-Pro Plus software with the wound healing tool (Media Cybernetics, Inc. Rockville, USA). Gap closure (%) = [Gap area (T0 − T)/Gap area T0] × 100% (where T is the image taking time and T0 is the time that the gap was initiated).

2.3.2 Time-Lapse Microscopy

The migration of surface microstructure preconditioned cells was tracked using a phase contrast time-lapse imaging microscope (IX81 motorized inverted microscope, Olympus, Hamburg, Germany) combined with a bold line cage incubator providing a humidified atmosphere (37 °C, 5% CO_2). Briefly, suspended cells collected from the different surfaces were reseeded in 24 well TCPs at a density of 3×10^4 cells per cm^2. After overnight adhesion, cells were observed and recorded every 10 minutes for 20 h. The images were processed using ImageJ software (National Institutes of Health, USA) combined with the software plug-ins "manual tracking" and "chemotaxis and migration tool" (ibidi GmbH, Martinsried, Germany) to calculate the migration parameters.

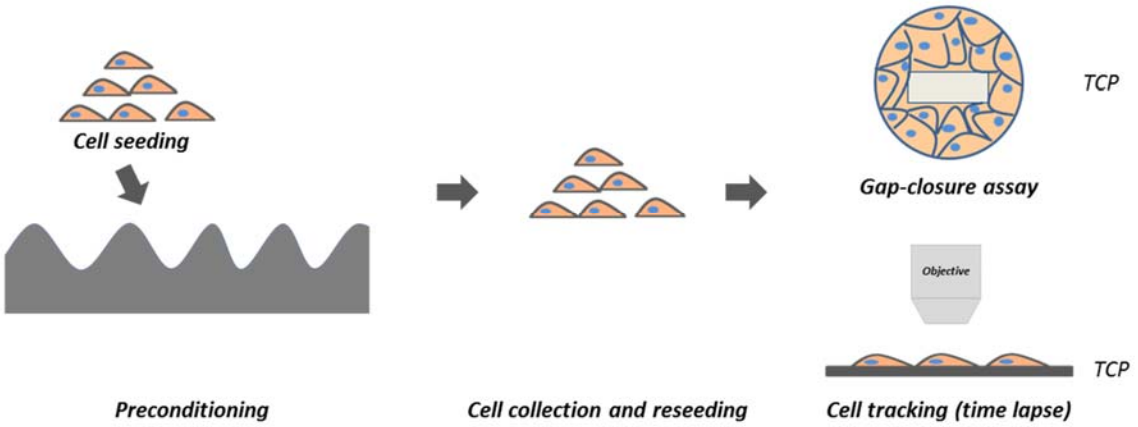

Fig. 1. Schematic diagram of the methods for investigating migration capacity of microtopography preconditioned hADSCs.

2.4 Immunocytochemistry

hADSCs cultured on different surfaces or reseeded in TCP and allowed to attach overnight were fixed by adding 4% (w/v) paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes and then permeabilized with 0.1% (w/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes. After blocking with 3% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) solution for 30 minutes, vinculin was stained with mouse anti-human primary antibodies (mouse anti-human vinculin monoclonal antibody (Merck Millipore, Darmstadt, Germany) and the Alexa Fluor® 488 labeled anti-mouse IgG antibody (Life Technologies, Darmstadt, Germany)); F-actin was stained with Alexa Fluor® 555 conjugated Phalloidin (Life Technologies, Darmstadt, Germany); the nuclei were stained with Hoechst 33342 (NucBlue® Live Reagent, Life Technologies, Darmstadt, Germany). After washing with PBS, the samples were scanned with a confocal laser scanning microscope (LSM 780, Carl Zeiss, Jena, Germany).

2.5 Protein extraction

hADSCs preconditioned on the microstructures for 14 days before and after overnight reseeding were lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) , and lysates supplemented with phenylmethylsulfonyl fluoride (Life Technologies, Darmstadt, Germany) and protease

inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation, the supernatant of cell lysates was stored at -80 ºC for further analysis.

2.6 Enzyme-linked immunosorbent assay (ELISA)

The expression levels of phosphorylated FAK (pFAK) were quantified by ELISA. The concentration of pFAK in the cell protein solutions were measured using the pFAK (Tyr397) ELISA kits (Life Technologies, Darmstadt, Germany). The total amount of proteins in the cell extract was determined using a BCA protein assay kit (Thermo Fisher Scientific, Bonn, Germany), and used to normalize the expression levels of the proteins of interest.

2.7 Western blotting

To quantify protein expression, the obtained protein solutions were denatured by heating at 95 °C for 5 minutes, separated by electrophoresis on 10% (v/v) SDS-PAGE and then transferred onto nitrocellulose membranes (Millipore, Darmstadt, Germany). The blots were probed with monoclonal primary antibodies (rabbit anti human MAPK, mouse anti human phospho-MAPK (Thr202, Tyr204), Millipore, Darmstadt, Germany) and IRDye 680LT and IRDye 800CW secondary antibodies (Li-Cor, Bad Homburg, Germany). Fluorescent signals were then detected using an Odyssey Imaging scanner and the intensity was analyzed by image studio software (Li-Cor, Bad Homburg, Germany).

2.8 Statistics

Experiments were repeated three times or more. For each experiment, cells were used between passage 3 to passage 5; 8 polystyrene inserts in each roughness level (24 inserts in three roughness levels) were used to collect the cell or protein samples. Data are shown as mean ± standard deviation. Statistical analysis was performed using the two-tailed independent-samples t-test, and a significance level (Sig.) < 0.05 was considered to be statistically significant.

3 Results

3.1 Surface characterization of polymer substrates

The topographic microstructures on the insert bottom were determined via optical profilometry. As previously described, R0 has a relatively plane surface with a *R*q value of 0.12±0.04 μm, while R1 and R2 have rougher surfaces (R1: 3.52±0.26 μm and R2: 16.04±1.24 μm) [35].

3.2 Migration of microtopography preconditioned hADSCs

The surface microstructure preconditioned cells were reseeded in a wound healing assay plate to perform the gap closure assay. The surface R1 and R2 preconditioned cells could more rapidly narrow down the gap when compared to the flat R0 surface preconditioned cells. The gap closure percentage of cells after 7 days preconditioning on the R1 surface was significantly higher than that of cells grown before on the flat surface R0 (Fig. 2A, B). To further confirm our findings, time-lapse microscopy was used to track cell migration and quantify migration velocity. The results strengthened the findings from the gap closure assay. Surface R1 preconditioned cells had a higher velocity than the cells from the other two surfaces. Already after 4 days preconditioning with different topographic microstructured surfaces, remarkable differences in velocity of hADSCs were observed (Fig. 2C, D).

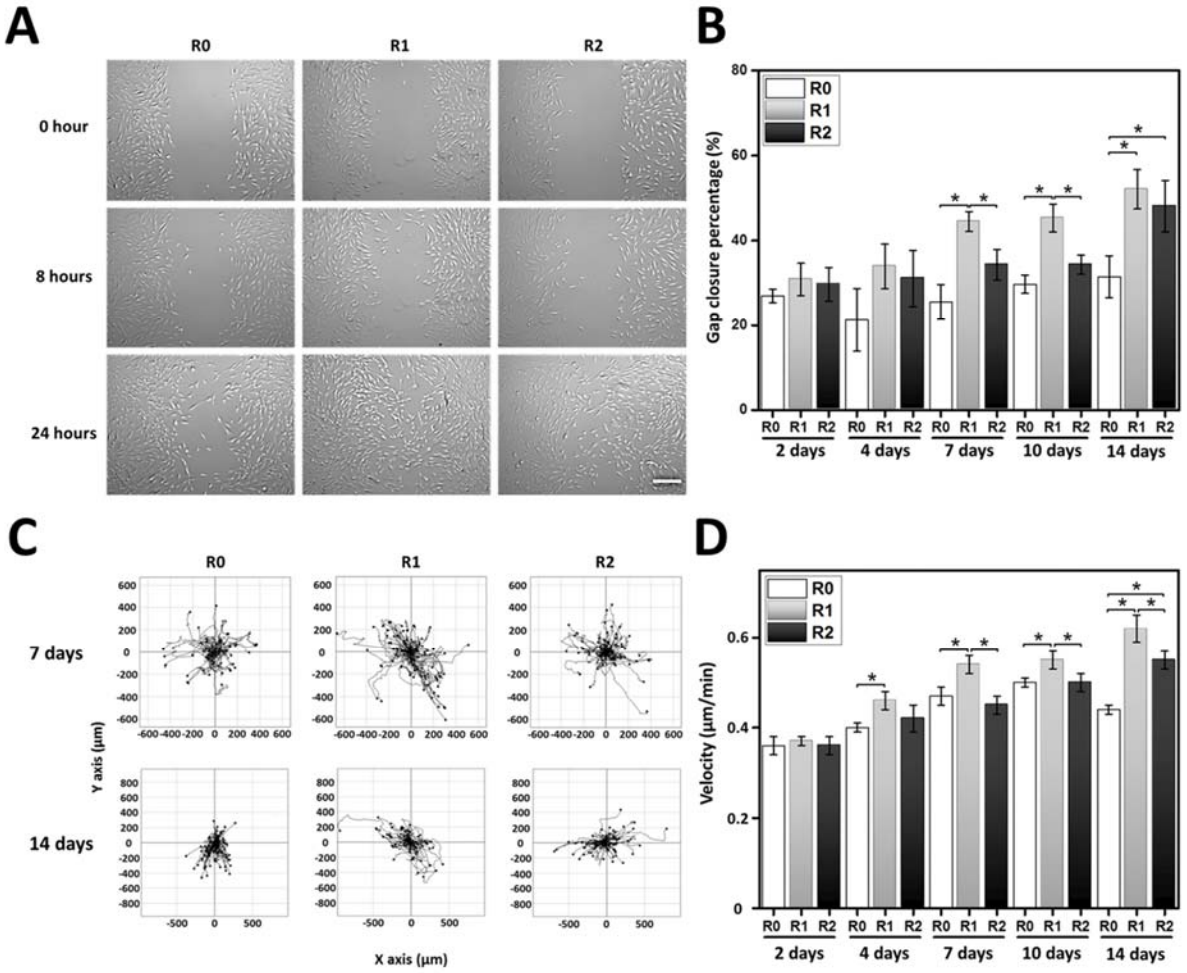

*Fig. 2. Enhanced migration capacity of hADSCs via surface microtopography preconditioning. (A) Representative images show the gap closure of hADSCs preconditioned for 7 days on different surface microstructures (bar = 500 μm). (B) The percentages of gap closure were quantified by Image-Pro Plus software (n = 5; *Sig < 0.05).* The hADSCs, preconditioned on different surfaces were reseeded on tissue culture plates up to 20 h and tracked to generate the migration trajectories (C) and to calculate the migration velocity *(D)(n≥50; Mean ± standard error of the mean (SEM); *Sig < 0.05).*

3.3 Focal adhesion of microtopography preconditioned hADSCs

Focal adhesion related components were investigated to study cell-material interactions. The focal adhesion complex formation was examined by immunofluorescent staining of vinculin and F-actin. After 4 days of cultivation, hADSCs on R1 and R2 surfaces formed smaller and more aggregated focal adhesions than those on the flat R0 surface (Fig. 3A). The 4 days preconditioned cells were reseeded on TCP. After overnight adhesion, the cells from R1 and R2 surfaces had more focal adhesions at the edges (Fig. 3B).

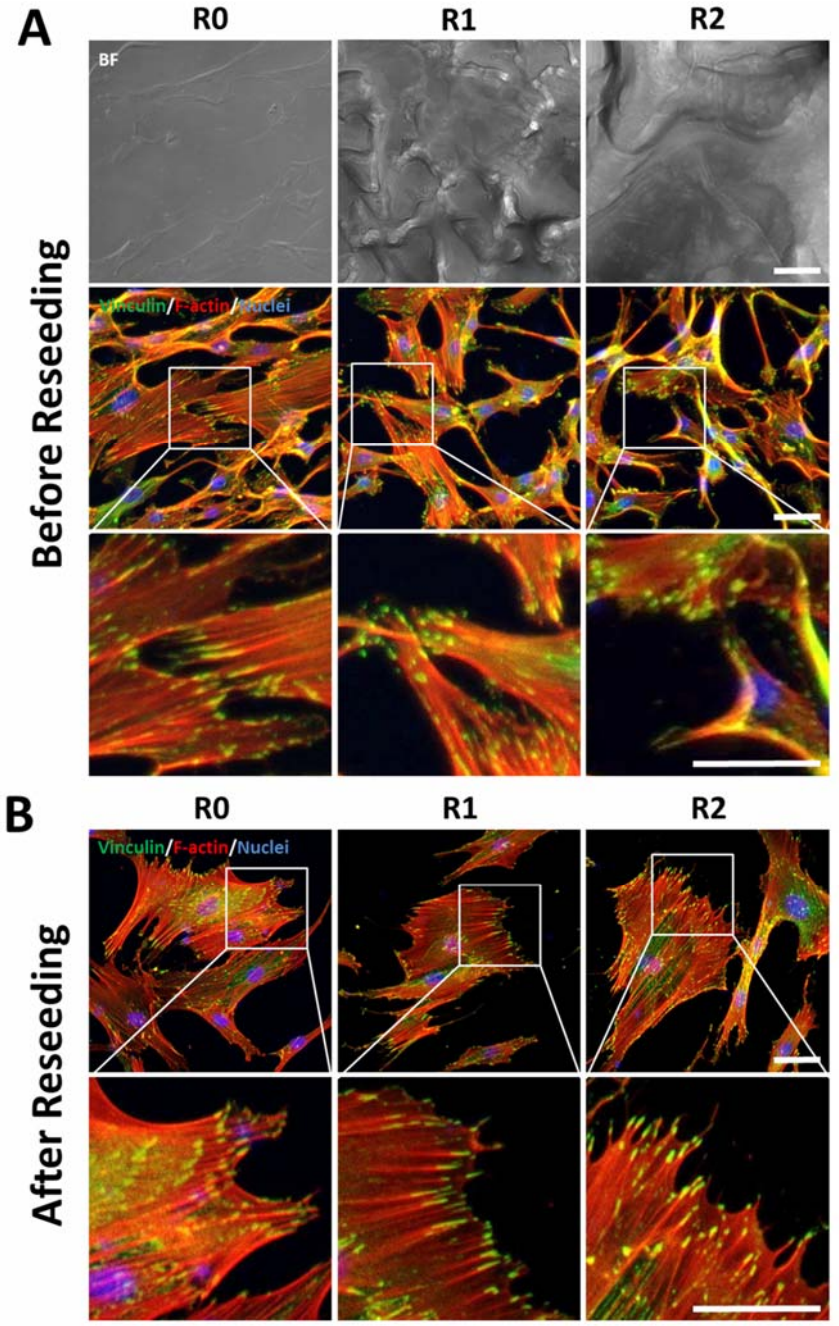

Fig. 3. Alterations in the appearance and number of focal adhesion of hADSCs in response to different topographic microstructures during and after preconditioning (BF: bright field). Representative laser scanning confocal microscopic images are shown: At day 4, the formation of focal adhesion and organization of F-actin cytoskeleton in hADSCs grown on three distinct

surfaces (A) and after reseeding on TCP (B) (red: F-actin; green: vinculin; blue: nuclei; bar = 50 μm).

3.4 FAK activation level of microtopography preconditioned hADSCs

After 4 days preconditioning, the pTyr397 FAK was significantly higher in the R1 group, and this elevated FAK phosphorylation level was maintained for up to 14 days (Fig. 4A). Further, after reseeding on TCP, a significantly increased FAK phosphorylation level of the cells from R1 surface was observed compared to the cells from R0 and R2 surfaces (Fig. 4B).

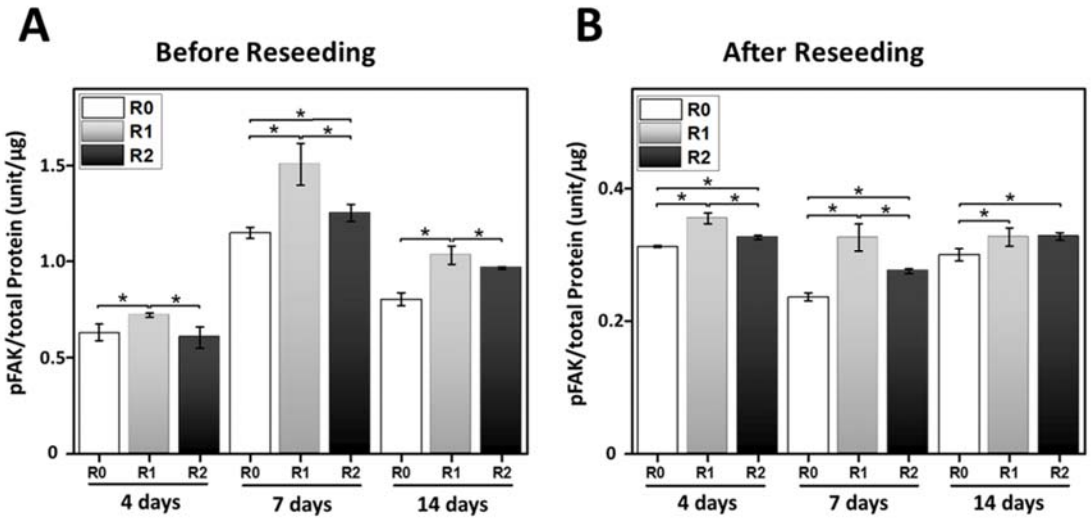

*Fig. 4. Activation levels of FAK of hADSCs during and after preconditioning. (A) The normalized FAK phosphorylation levels of hADSCs harvested directly from microscale roughness surfaces at day 4, 7 and 14 and (B) roughness pretreated cells post 24 h reseeding (n = 3; *Sig < 0.05).*

3.5 MAPK phosphorylation level of microtopography preconditioned hADSCs

Protein blotting was performed to evaluate the expression and activation of MAPK in 14 days surface microtopography preconditioned hADSCs before (Fig. 5A) and after reseeding (Fig. 5B). It was found that the ratio of phosphorylated MAPK to total MAPK was significantly higher in hADSCs grown on microtopographic surfaces R1 and R2 compared to that on flat surface R0 (Fig. 5C). After reseeding of preconditioned cells on TCP, the ratio of phosphorylated MAPK to total MAPK was sustained at a high level from cells preconditioned on a R1 surface (Fig. 5C).

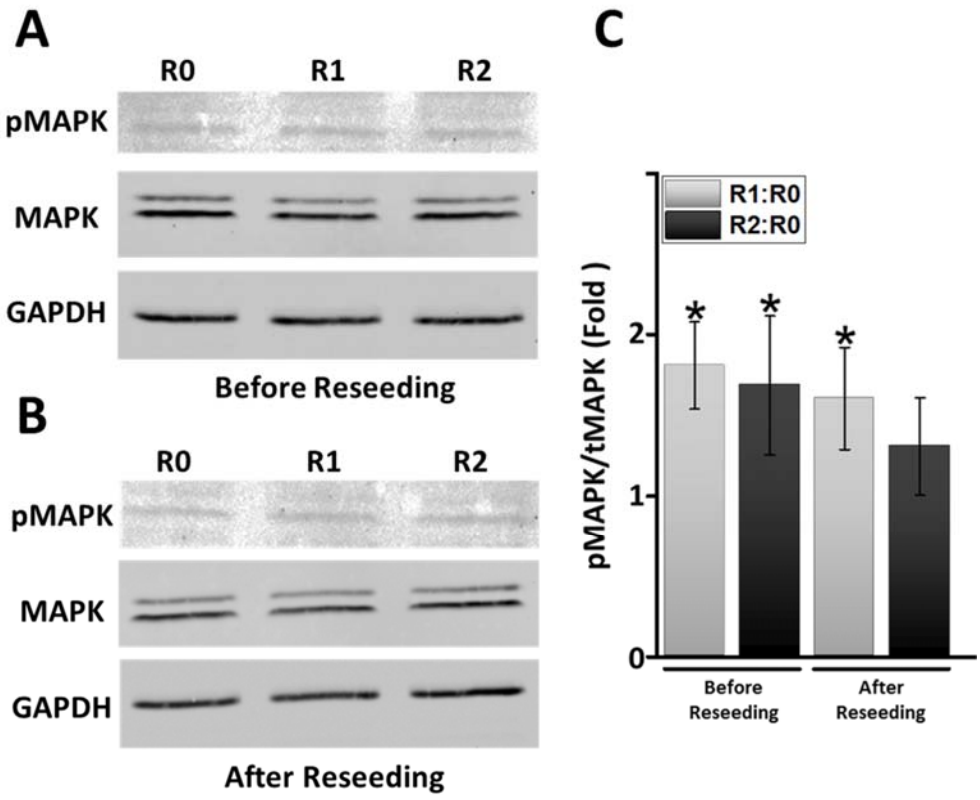

*Fig. 5. Surface microtopography enhanced MAPK phosphorylation. Expression of MAPK and phosphorylated MAPK in 14 days surface microstructure preconditioned cells before (A) and after reseeding on tissue culture plate (B) was analyzed via western blot. (C) The statistical analysis of pMAPK/to total MAPK (tMAPK) ratio are presented in a fold-change histogram (n = 3, * Sig. < 0.05 compared with R0).*

4 Discussion

It has been reported that stem cell behavior can be influenced by preconditioning in micro environments [38]. In this study, microstructures were applied to precondition hADSCs. The preconditioned cells were then harvested and reseeded in the same conventional cell culture vessel to compare the migration capacity and activity of potentially migration-associated modulators such as FAK and MAPK. Our results demonstrated that the hADSCs were able to develop different migration capacities induced by preconditioning on different topographic microstructures. Within these microstructures, the cell-material contact surface with intermediate roughness level promoted cell migration. In parallel to the increase in migration, an elevated FAK and MAPK activation level was observed. These findings indicate that the MSCs are capable of preserving the previously received structural signals. Only the cells preconditioned for 4 days or longer acquired and maintained the enhanced migration capacity, which suggests that the functional alteration of MSC behavior may only be triggered upon exposure to a certain threshold dosage of topographic cues. This phenomenon is consistent with a recent report, which illustrated a clear relationship between the stem cell memory and the precondition dosage of mechanical signals [39].

The formation and turnover of focal adhesion (FA) complexes are crucial dynamics in cells migration [40]. During migration, on one hand, FA forms at the leading edge so as to initially generate the necessary forces to pull the cell body forward. On the other hand, FA turns over at the rear edge to eliminate the resistance force and continue cell movement. Hence continuously synchronized formation and turnover of FA of the cell body are required in migration [41]. Moreover, it has been demonstrated that the size of FA is predictive for the cell migration speed [42]. In the present study, the distinct appearance in size and density FA of cells during and after microstructure preconditioning might contribute to the initiation of the different migration.

As one of the most prominent signaling molecules, FAK is involved in both formation and turnover of FAs during cell migration [32,43]. FAK is composed of an N-terminal FERM domain, a C-terminal FAT and an intermediate kinase domain. The FERM domain of FAK has been demonstrated to interact with integrins and growth factor receptors [44]. These extracellular interactions can elicit the intracellular autophosphorylation of FAK at Tyr397, located at the kinase domain. The phosphorylated FAK at Tyr397, which binds and recruits Src protein, can lead to

further phosphorylation of FAK at various tyrosine sites including Tyr576, Tyr577, Tyr861 and Tyr925 [45-47]. In terms of dynamics in FA, on one side, Tyr925 is necessary for the interaction of FAK with Grb2, which helps the recruitment of dynamin to adhesion site to support FA formation [48]. On the other side, FA disassembly requires the dephosphorylation of phosphorylated Tyr397 and FAK after the extension of microtubules to FA [48,49]. Therefore, activation of FAK is indispensable in formation, turnover of FA, and intracellular transduction of integrin and growth factor receptor mediated external signals. It has been demonstrated that cells with highly activated FAK level exhibited an enhanced migration capacity [50-52]. Here, in agreement with these studies, an increased cell migration speed accompanied with an elevated FAK phosphorylation level at Tyr397 site was detected in hADSCs that have been preconditioned with R1 microstructured PS surface. It is suggested that the enhanced cell migration through microstructure preconditioning might be closely associated with the elevation of FAK activity.

MAPK regulates the cell migration through the FAK and myosin light chain kinase. The activation of MAPK can induce the phosphorylation of myosin light chain, which could enhance the cell migration [53]. Further, the activation of FAK was reported to subsequently activate its downstream effectors including MAPK [54]. Our results demonstrated that the R1 surface microstructure could enhance migration capacity, and elevated both FAK and MAPK phosphorylation levels. This alteration of subcellular structure and function of cells is in consistence with previous studies [55,56], in which it was demonstrated that the formation of a paxillin-FAK-MAPK complex regulates the cell migration capacity. Therefore, we assume that topographic microstructures modulate the MSCs migration capacity through the activation of FAK and MAPK.

In summary, the observations presented here indicate that hADSCs migration capacity could be modulated by surface microstructures. The appropriate roughness level promoted migration, which was accompanied with higher levels of activated FAK and MAK. This might be helpful for further understanding the underlying mechanism of microstructure-modulated hADSCs migration capacity.

5 Conclusion

Effects of surface microtopography on hADSCs migration capacity were investigated in this study. The cells preconditioned with topographic microstructures exhibited distinct morphologies, focal adhesion sizes and migration velocities. The appropriate topographic microstructure significantly enhanced the cell migration capacity, and this effect was accompanied with increased FAK and MAPK activation level. These findings suggest that the cell migration capacity could be modulated by the preconditioning of cells via surface topographic microstructures of culture vessels, which might be helpful for improving the *in vivo* stem cell therapeutic efficacy by fine-tuning the *in vitro* physical or mechanical cues prior to stem cell transplantation.

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