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Microscale roughness regulates laminin-5 secretion of bone marrow mesenchymal stem cells

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Abstract:

Laminin-5 (Ln-5), an important extracellular matrix (ECM) protein, plays a critical role in regulating the growth and differentiation of mesodermal tissues, including bone. Ln-5 can be secreted by the mesenchymal stem cells (MSCs), and Ln-5 promotes MSCs osteogenic differentiation. It has been demonstrated that a substrate`s surface topography could regulate MSC secretion and differentiation. A better understanding of the mechanism of how Ln-5 and surface roughness regulate MSC osteogenic differentiation would guide the design of surface topography and coatings of orthopedic implants and cell culture substrates. However, few studies have investigated the relationship between surface roughness and the secretion of Ln-5 in MSC osteogenic differentiation. Whether substrate surface topography regulates MSC differentiation via regulating Ln-5 secretion and how surface topography contributes to the secretion of Ln-5 are still not known. In this study, the influence of microscale roughness at different levels (R0, R1 and R2) on the secretion of Ln-5 of human bone marrow MSCs (hBMSCs) and subsequent osteogenic differentiation were examined. hBMSCs spreading, distribution and morphology were greatly affected by different roughness levels. A significantly higher level of Ln-5 secretion was detected on R2, which correlated to the local cell density regulated by the rough surface. Ln-5 binding integrins (α2 and α3) were strongly activated on R2. In addition, the results from hBMSCs on R0 inserts with different cell densities further confirmed that local cell density regulated Ln-5 secretion and cell surface integrin activation. In addition, the mineralization level of MSCs on R2 was remarkably higher than that on R0 and R1. These results suggest that hBMSC osteogenic differentiation level on R2 roughness was enhanced via increased Ln-5 secretion that was attributed to rough surface

regulated local cell density. Thus, the microroughness could serve as effective topographical stimulus in cell culture devices and bone implant materials.

Key words: mesenchymal stem cells, roughness, cell density, laminin-5, osteogenesis

1. Introduction

Extracellular matrix (ECM), as stem cell niche component, is a critical player for stem cell differentiation. The chemical composition, mechanical properties, topography, and density of ECM are major factors greatly regulating stem cell adhesion, migration, proliferation and differentiation [1-5]. Laminin (Ln) family proteins are components of ECM, which are mainly located in the basement membrane of many epithelial and endothelial tissues. Ln mediates cell adhesion, migration, proliferation and differentiation via interaction with cell surface integrins, which further influence tissue organization [6,7]. Each Ln molecule is composed of an α, β and γ subunit. Among all the Ln isoforms, Ln-5 is typically found in the tissue derived from endoderm, ectoderm and mesoderm [8-10] and is composed of α3, β3 and γ2 subunits [11]. Ln-5 has multiple functions in regulating vascular smooth muscle cells, growth and migration as well as promoting mesenchymal stem cell (MSCs) osteogenic differentiation [12]. MSCs are capable of secreting Ln [13]. Integrin α3 and β1 of hMSCs can bind specifically to Ln-5, and further activate its downstream RhoA-ROCK and MAPK/ERK signal pathways. The cell contractility is subsequently enhanced via elevation of myosin light chain (MLC) activation [14-16], which promotes the osteogenic differentiation of hMSCs.

Bone marrow mesenchymal stem cells (BMSCs) are multipotent stem cells with high potential to differentiate into osteocytes [17], which makes them valuable as a cell source for treating bone diseases [18]. The potential and efficiency of BMSC osteogenic differentiation are highly regulated by their microenvironment, composed of multiple factors including chemical, physical and biological factors [19]. Cell density is reported as an effective factor to regulate MSC behaviors like proliferation and differentiation [20,21]. On 2D substrates, the appropriate cell density could enhance alkaline phosphatase expression and Ca^{2+} mineralization of MSCs [22]. In 3D environments, the appropriate cell seeding density of MSCs promotes the formation of cell aggregation that further enhances their osteogenic potential and represses adipogenic potential in gene expression level [23]. Moreover, the appropriate cell density can enhance the synthesis and secretion of ECM proteins such as collagen [24,25].

The substrate surface topography is another important factor that influences the osteogenic differentiation of BMSCs. The efficacy of promoting osteogenic differentiation of MSCs via fabricating cell substrates with surface roughnesses of different scale levels was studied [26]. Substrate surface topography and Ln-5 play important roles in inducing MSC osteogenic differentiation. The understanding of how the surface topography and Ln-5 regulate MSC osteogenic differentiation would provide new insight for the design of surface topographies and coatings of orthopedic implants and cell culture substrates. Based on these previous studies, we hypothesize that an optimized surface roughness might influence MSCs osteogenic differentiation via regulation of cell density and subsequent Ln-5 secretion.

Polycarbonate (PC) cell culture inserts with three types of distinct roughnesses levels at the bottom surfaces were prepared in order to verify our hypothesis. The same number of hBMSCs were seeded on the different inserts. The distribution of cells, Ln-5 secretion, and cell surface integrin expression levels as well as osteogenic differentiation levels of the cells on different surfaces were examined. Additionally, hBMSCs were seeded on smooth surface inserts with a range of different cell densities, Ln-5 secretion and integrin expression levels of cells were characterized, and the relationship between cell density and Ln-5 secretion was investigated.

2. Materials and methods

2.1. Materials for cell culture

Polycarbonate (PC, trade name Makrolon® 2805, Bayer, Germany) inserts with a suitable size to fit the standard 24-well tissue culture plate (TCP) were prepared via injection molding [27]. Three differently structured modules were utilized to fabricate the inserts with different types of bottom surface topography: a module with a polished contact surface (R0), and two modules with micro-structured surfaces according to the norm DIN 16747: 1981-05, M30 (R1) and M45 (R2). The microscale topography of the insert bottom was characterized with an optical profilometer type MicoProf 200, and analyzed with the software AQUIRE (ver. 1.21) and the software MARK III (ver. 3.9) according to the method previously reported [28]. The parameters of surface roughness are the following: R0 (smooth surface as control, arithmetic average roughness (Ra) 0.05 ± 0.01 μm), R1 (Ra 2.94 ± 0.11 μm, average peak spacing (RSm) 160.3 ± 8.2 μm) and R2 (Ra 11.30 ± 0.43 μm, RSm 279.3 ± 32.3 μm). The inserts were sterilized by gas sterilization (gas phase: 10% (v/v) ethylene oxide, 54 °C, 65% relative humidity, 1.7 bar, 3 hours of gas exposure time and 21 hours of aeration phase).

2.2 Characterization of cell morphology and cell distribution

hBMSCs (SCC034, Merk Millipore, Darmstadt, Germany) were seeded into inserts with a density of 2×10^4 cells/insert and cultured in MesenPRO RS™ growth medium (GM) (ThermoFisher Scientific, Waltham, USA) for 4 days at 37 °C, in a humidified atmosphere containing 5% (v/v) CO_2. Then cells were fixed with 4% (w/v) paraformaldehyde (Sigma–Aldrich, St. Louis, MO USA) and permeabilized with 0.1% (v/v) Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA) followed by blocking with 3% (w/v) BSA buffer. F-actin was stained with ActinRed™ 555 Ready Probes (Life Technologies, Darmstadt, Germany). Cell nuclei were stained with Hoechst 33342 (Life Technologies, Darmstadt, Germany). The secretion of ECM (Ln-5 and fibronectin) were characterized by rabbit anti-Ln-5 alpha 3 antibody (Abcam, Cambridge, United Kingdom). Anti-rabbit IgG (H+L)-Alexa Fluor® 633 (Invitrogen, California, USA) were used as secondary antibodies. Samples were visualized and captured by confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena, Germany). Cell distribution and local cell density were analyzed using Image J software (National Institutes of Health, USA).

2.3 Ln-5 secretion

For the study of Ln-5 secretion of hBMSCs on surfaces with different roughnesses, hBMSCs were seeded with a density of 2×10^4 cells/insert. For studying the relationship between cell density and Ln-5 secretion, hBMSCs were seeded on R0 with a range of different cell densities (2×10^4 cells/insert, 1×10^4 cells/insert and 5×10^3 cells/insert). The cells were cultured in GM for 4 days. The secreted Ln-5 was characterized by immunofluorescence staining, which has been described in section 2.2. The quantification of Ln-5 secretion on different surfaces was examined by human Ln-5 ELISA kit (Cusabio, Wuhan, China). The amount of total protein in the cell extraction was determined using a BCA protein assay kit (Thermo Fisher Scientific, Bonn, Germany).

2.4 Characterization of cell surface integrin activation and aggregation

hBMSCs (2×10^4 cells/insert) were seeded on the inserts with different surface roughness levels, and a range of different cell densities (2×10^4 cells/insert, 1×10^4 cells/insert and 5×10^3 cells/insert) of cells were seed on the smooth surface inserts. The cells were cultured in GM for 4 days and then harvested, fixed with 4% (w/v) paraformaldehyde solution (Sigma-Aldrich, St. Louis, MO, USA), and blocked with 3% (w/v) BSA (Sigma-Aldrich, St. Louis,

MO, USA) solution. After that, the cells were stained with anti-human integrin alpha-2 (Abcam, Cambridge, UK) and anti-human activated integrin alpha-3 (Abcam, Cambridge, UK) primary antibodies, and Alexa Fluor® 488-labeled IgG and Alexa Fluor® 633-labeled IgG (Life Technologies, Darmstadt, Germany) second antibodies. The stained cells were analyzed by flow cytometer (MACSQuant®, Miltenyi Biotec, Bergisch Gladbach, Germany). Data analysis was performed using "Flowjo" software (Tree Star Inc., Ashland OR, USA).

2.5 Osteogenic differentiation assay

hBMSCs were seeded on the surface with different roughnesses at a density 2×10^4 cells/ insert, and cultured in GM for 4 days. Thereby, osteogenic induction medium was used to culture the cells for 21 days. Cells were fixed with 4% (w/v) paraformaldehyde. Alizarin red S (ARS) (Sigma-Aldrich, St. Louis, MO, USA) solution was used to stain mineralization deposition according to the protocol, which was reported previously in [29]. The stained samples were visualized and captured by inverted light microscopy (Axiovert 40C, Carl Zeiss, Jena Germany). ARS was extracted from the stained samples by acetic acid and the absorbance was measured at 405 nm [29] for the quantification of hBMSCs osteogenic differentiation. The absorbance values were normalized by cell number that was determined using CCK-8 kit (Thermo Fisher Scientific, Bonn, Germany) according to the manufacturer protocol.

2.6 Statistics

The number of replication for quantitative experiments was equal to or larger than three as indicated respectively in the figure legends. Unless indicated otherwise, the data were expressed as arithmetic mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA with post hoc Tukey HSD test. * P< 0.05 was considered to be statistically significant

3. Results

3.1 hBMSCs morphology on the surface with different roughnesses

Immunofluorescent staining was performed on the surface with different roughness levels to examine the cell adhesion and morphology. MSCs were able to adhere and spread well on PC surfaces (Fig.1). The cells on R0 surface showed the typical spindle-shape with highly aligned actin cytoskeleton. In contrast, the cells on R1 and R2 were less aligned and they displayed

poorly oriented stress fibers. The results indicated that the R1 and R2 surfaces regulated hBMSCs shape and orientation effectively.
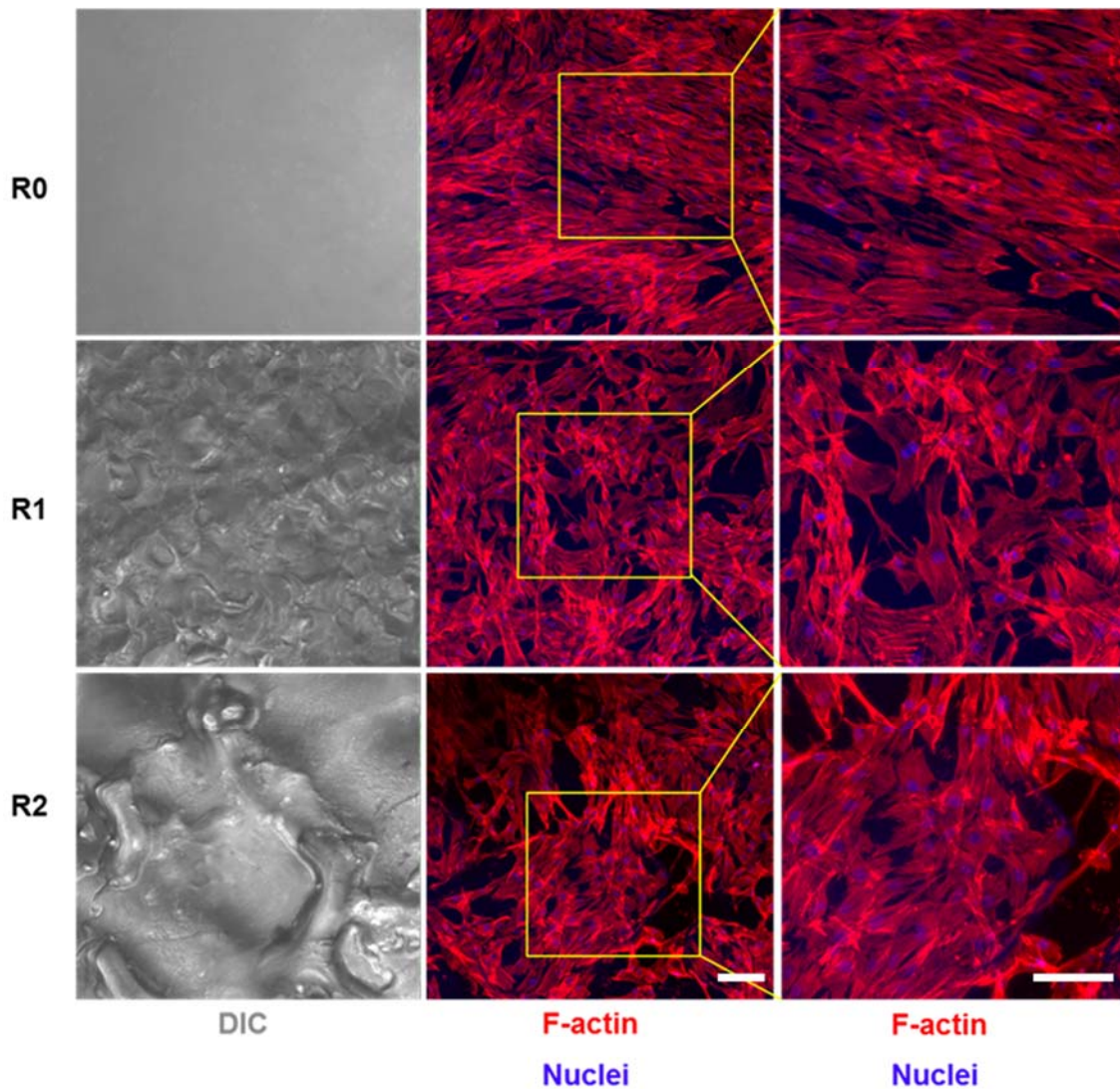

Fig. 1: Top-view of hBMSCs cultured on surfaces with different microroughnesses. The hBMSCs were cultured on different surfaces for 4 days followed by fluorescence staining to visualize the actin cytoskeleton (red) and nuclei (blue). (Scale bar = 100 μm).

3.2 Cell distribution and Ln-5 secretion

Distribution of hBMSCs and Ln-5 secretion on the surfaces with different roughness levels were investigated via fluorescence staining. The distribution of cells on the R0 surface was more uniform than that on R1 and R2 surfaces. Especially on the R2 surface the distribution of cells was highly dependent on the local surface topography with higher cell density in the "valley" area and lower density in the "plateau" area (Fig. 2a). The quantitative analysis

showed that the cell densities of hBMSCs on R0 and R1 were 595 ± 79 cells/mm^2 and 577 ± 68 cells/mm^2, respectively. There was no significant difference in cell density on R0 and R1 surfaces. However, on the R2 surface, the local cell density (white lines enclosed areas) reached 925 ± 127 cells/mm^2, which was significantly higher than that on R0 and R1 surfaces. The rough surface influences Ln-5 secretion besides cell distribution (Fig. 2a). The Ln-5 secretion on the R2 surface showed a similar pattern with the cell distribution. The Ln-5 fluorescence intensity of the local area with high cell density (white lines enclosed areas) was higher than that of other areas. In addition, the Ln-5 fluorescence intensity on R2 surface was stronger than that on R0 and R1 surfaces. The quantitative analysis results showed that the Ln-5 secretion of cells cultured on R2 surface was significantly higher than that on R0 and R1 surfaces (Fig. 2b).
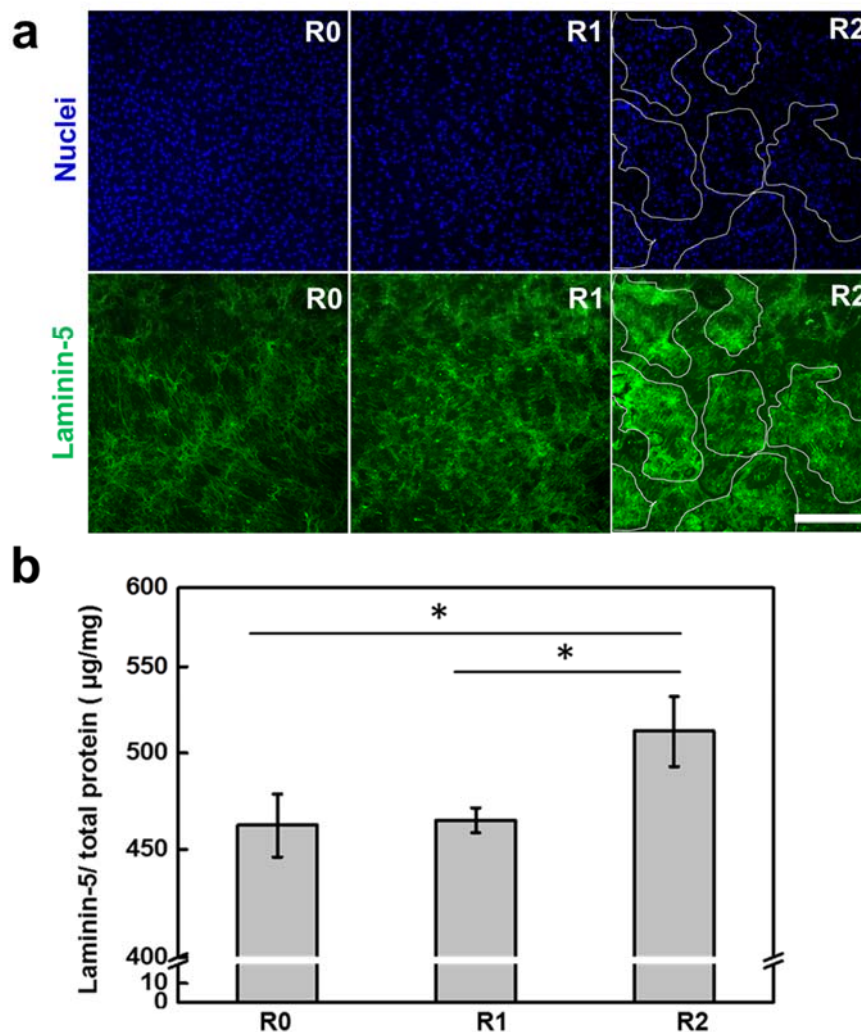

Fig. 2: Distribution and Ln-5 secretion of hBMSCs on surfaces with different roughnesses. hBMSCs were cultured on surfaces for 4 days followed by fluorescence staining to visualize the nuclei (blue) and Ln-5 (green). a: Top-view of hBMSCs and Ln-5 distribution on surfaces with different micro

roughnesses; (scale bar = 200 μm). Inhomogeneous cell and Ln-5 distribution were observed on R2 surface, as indicated by the areas enclosed by the white lines (high cell density). b: Quantitative analysis of Ln-5 secretion of cells that were cultured on the surface with different roughnesses. (n = 3; *Sig < 0.05).

3.3 Cell surface integrin activation and aggregation.

Cell surface integrins were characterized to investigate the interaction between cells and substrate surfaces with different roughness levels. The cells cultured on R2 surface presented significantly higher integrin α2 (total) expression levels than that of cells on R0 and R1 surfaces (Fig. 3a). The characterization of activated integrin α3 presented a similar result (Fig. 3b). These results suggested that R2 surface roughness enhanced cell surface integrin activation and aggregation.
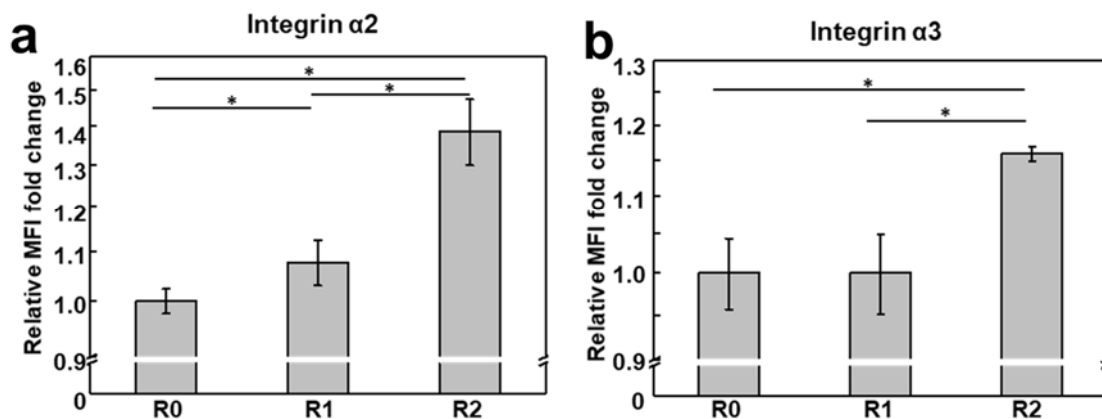

Fig. 3: Surface roughness regulated Ln-5 secretion further influenced integrin activation and aggregation. hBMSCs were cultured on different surfaces for 4 days and then detached and fixed, the integrin levels were characterized by flow cytometry. a: Relative mean fluorescence intensity (MFI) fold change of cell surface total integrin α2 compare to that of cells on R0; b: Relative MFI fold change of cell surface activated integrin α3 compared to that of cells on R0. (n = 4; * p < 0.05).

3.4 Assay of Ln-5 secretion and integrin with different cell densities

The influence of cell density on secretion of Ln-5 and integrin α2 and α3 was studied via the evaluation of Ln-5 secretion and integrin α2 and α3 levels of cells cultured in different cell density. The high cell density group (2×10^4 cells/insert) presented a significantly higher fluorescence intensity than that of the low cell density groups (1×10^4 cells/insert and 5×10^3 cells/insert) (Fig. 4a). The quantitative analysis of Ln-5 secretion indicated that there was a

remarkably higher Ln-5 secretion level of cells at the high cell density than that of cells at low cell density (Fig. 4b), which is consistent with the result of immunofluorescence staining. A significantly higher expression level of total integrin α2 and activated integrin α3 was found at a high cell density compared to that at a low cell density (Fig. 4c and d). These results indicated Ln-5 secretion of hBMSC was regulated by controlling the initial cell seeding density.
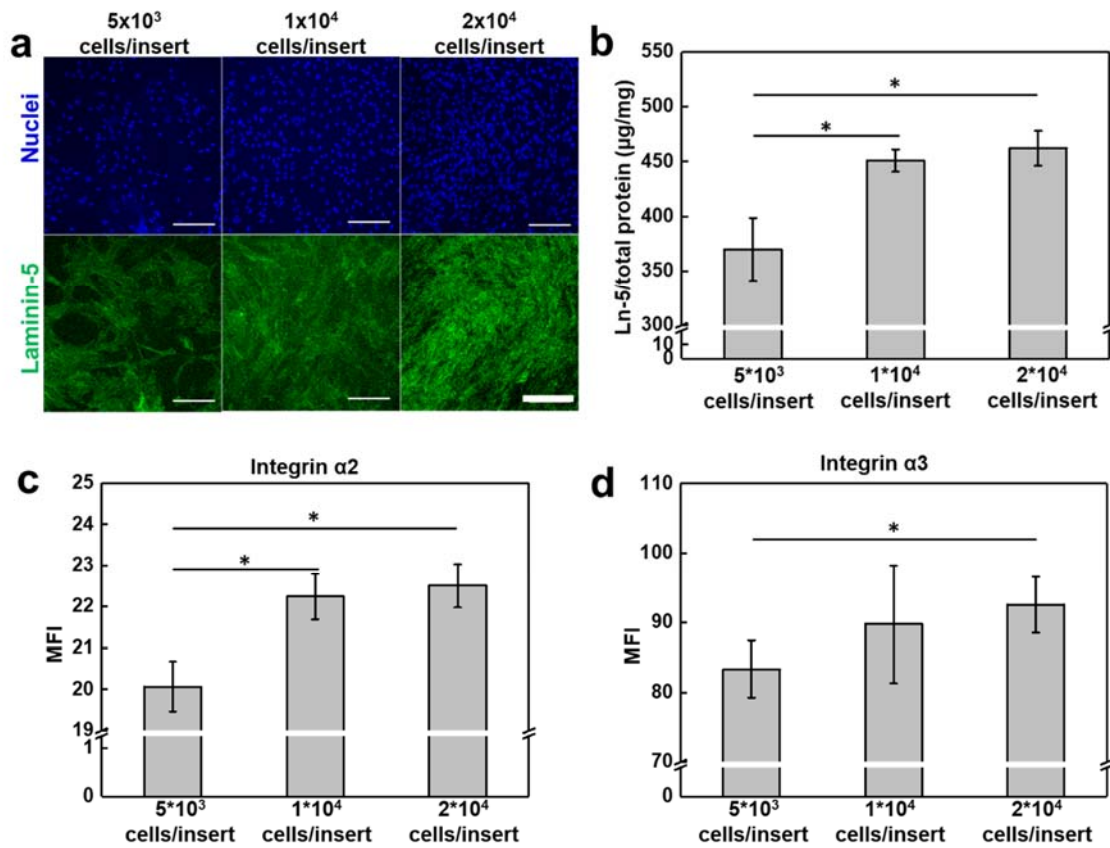

Fig. 4: Cell density regulated Ln-5 secretion and cell surface integrin expression level. a: hBMSCs were cultured on R0 surface for 4 days followed by fluorescence staining to visualize the nuclei (blue) and Ln-5 (green), (scale bar = 200 μm); b: Quantification of Ln-5 secretion of cells with different cell densities by ELISA. c: MFI of total integrin α2 on cell surface; d: MFI of active integrin α3 on cell surface. (n = 4; * $p < 0.05$).

3.5 Osteogenic differentiation assay

The osteogenic differentiation of hBMSCs on the surfaces with different roughnesses levels were examined by the ARS staining. hBMSCs seeded on R2 surface presented a significantly higher osteogenic differentiation level than that of hBMSCs on R0 and R1 surfaces (Fig. 5).
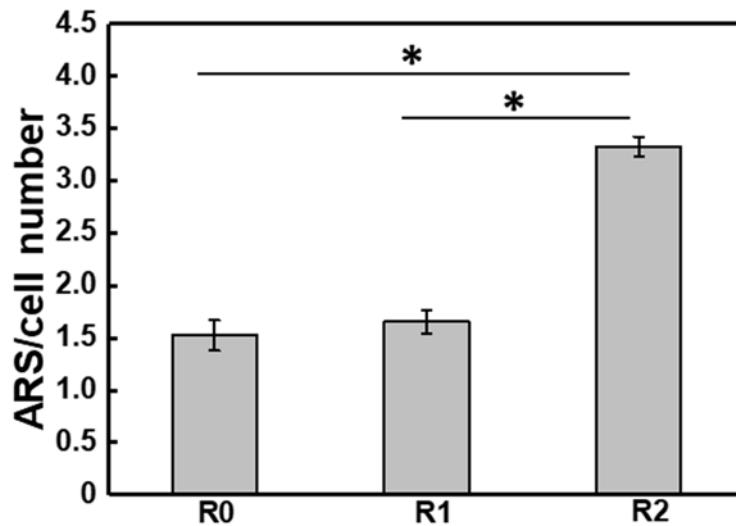
Fig. 5: Roughness of PC surface regulates hBMSCs osteogenic differentiation. hBMSc were cultured on the surfaces with different roughnesses in osteogenic induction medium for 21 days, the osteogenic differentiated cells were stained by Aliza Red S (ARS) solution. Then the stained ARS was extracted by acetic acid and analyzed semi-quantitatively. (n = 6; * p < 0.05).
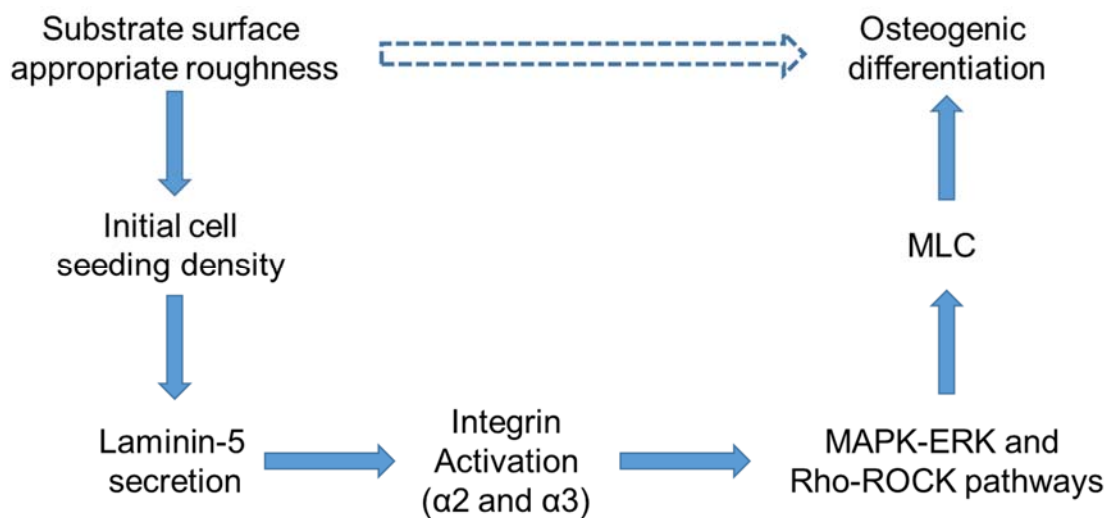

Fig. 6 A simplified model of surface roughness regulating the osteogenic differentiation of MSCs. MAPK: mitogen-activated protein kinases; ERK: extracellular signal-regulated kinases; Rho: Rho guanine nucleotide exchange factors; ROCK: Rho-associated protein kinase; MLC: myosin light chain.

4. Discussion

In this study, we designed and developed a PC surface system with different microscale roughnesses levels and showed that fine-tuning of the surface roughness could enhance

hBMSC osteogenic differentiation *in vitro*. The fine-tuning of the surface roughness influenced the initial cell seeding density, which further affected the Ln-5 secretion. The enhanced secretion of Ln-5 resulted in the promotion of the cell surface integrin α2 and α3 aggregation and activation, and subsequently, regulated osteogenic differentiation level of hBMSCs.

In the development of biomaterials for defect tissue regeneration and repair, the physical structure can directly regulate the seeding cell distribution in the scaffold, which influences the cell-cell interaction and other cell behaviors such as proliferation and differentiation [30,31]. In this study, we found that the R2 surface roughness regulated the cell distribution. On R2 surface, the local cell density was significantly higher than that on R1 and R0 surfaces, which could promote the cell-cell interaction. The enhancement of cell-cell interaction on 2D surface and 3D microenvironments with appropriate high cell accumulation level promoted MSCs osteogenic differentiation has been demonstrated [23,32]. Numerous studies about the effect of cell density on cell behaviors found that there was an optimal cell density for stem cell osteogenic differentiation. If the cell densities were below the optimal density, cell bone markers expression levels and ECM production could be promoted by increasing the cell seeding density. However, when the cell density exceeded the optimal density, the abilities of cells such as proliferation, migration and differentiation would decrease [33-36]. This statement is consistent with the research result of McBeath *el. at* [37], in which exceeding the optimal high cell density depressed MSC osteogenic differentiation due to limited space for cell spreading that results in decreasing cytoskeleton tension. In our study, the cell seeding density was below the optimal density, after 4 days cultivation the cell confluency did not reach 100% and did not cover the substrate surface completely (Fig. 1).So the R2 rough surface regulated local high cell density would promote MSC osteogenic differentiation (Fig. 5).

The local appropriate high cell density on R2 surface also enhanced the ECM production. Ln-5 secretion in the area of the high cell density on R2 surface was significantly higher than that on R1 and R0 surfaces. Ln-5 is a member of the LN family, which are components of cell-secreted ECM and mediates cell attachment, migration, and tissue organization [6]. Ln-5 is specifically bound by α2β1, α3β1, α6β1 and α6β4 integrin receptors [38], all of which are found in hMSCs [39]. The increased density of ECM results in the elevation of cell surface integrin activation, which is consistent with the results of the cells on R2 surface presented remarkably higher levels of integrin α3 activation and total integrin α2 compared to cells on R0 and R1 surfaces (Fig. 3). hBMSCs were cultured with different cell densities, Ln-5 secretion and cell

surface integrin expression of cells were characterized. The results (Fig. 4) confirmed that the cell density was a key factor to regulate ECM secretion and cell surface integrin activation and aggregation. The activated and aggregated integrin clusters are concentrated in specialized organelles known as focal adhesions, which are also enriched with bundles of actin and associated cytoskeletal proteins, including vinculin, talin, paxillin and tensin to form focal adhesion [40], and further activate the downstream RhoA-ROCK pathway to enhance cell contractility, which promotes stem cell osteogenic differentiation [14]. hMSCs cultured on Ln-5 stimulated global changes in osteogenic genes/proteins expression in hMSC and resulted in the commitment of these cells to the osteogenic phenotype [12]. Ln-5 activated cell surface integrin α3β1 receptor, which activates extracellular signal-related kinase (ERK) [15,16]. Furthermore integrin α2 upregulated expression promoted hMSCs osteogenic differentiation via FAK-ERK1/2 pathway [41]. ERK is a member of mitogen-activated protein kinase (MAPK) family that stimulates the osteogenic differentiation of hMSCs via phosphorylation of the osteogenic transcription factor Runx2/CBFA-1[42]. Bone morphogenic proteins (BMPs) are osteoblast-specific transcription factors that bind to chordin like cysteine-rich repeats that are found in several ECM proteins [43]. Since the α3 and γ2 chains of Ln-5 contain potential cysteine-rich regions that could bind BMP, Ln-5 acts as a repository for BMPs, and other growth factors may interact with Ln-5 and/or its associated proteins.

In summary, these data suggested that substrate surface roughness regulated the initial cell seeding density, and then affected Ln-5 secretion, which further modulated the osteogenic differentiation of hBMSCs (Fig. 6). In the procedure of cell seeding on substrate, surface topography (roughness) regulated initial cell seeding density, the appropriate cell density enhanced cell proliferation and ECM (Ln-5) secretion. The incensement of Ln-5 secretion promoted integrin α2 aggregation and integrin α3 activation of hBMSCs surface, which activated focal adhesion kinase (FAK) and downstream (MAPK-ERK and Rho-ROCK) pathways to enhance cell contractility [44], and subsequently enhanced the osteogenic differentiation of hBMSCs.

5. Conclusion

In this study, we investigated the influence of substrate surface roughness on the enhancement of hBMSC osteogenic differentiation efficacy. We found that the appropriate scale surface roughness regulated initial cell seeding density, the appropriate high cell density affected Ln-5 secretion. The increased local Ln-5 density enhanced the activation of cell surface integrin

and further activated its downstream pathway, subsequently promoting the osteogenic differentiation of hBMSCs. These findings allow a better understanding of MSC response to the physical structures of a scaffold. Optimizing the micro-roughness of orthopedic implant materials surface to promote osteogenic differentiation of hBMSCs is an effective method for bone repair.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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