

Mesenchymal Stem Cells Proliferation on Konjac Glucomannan Microcarriers: Effect of Rigidity

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Abstract Mesenchymal stem cells (MSCs) have attracted great attention as a source of cells for regenerative medicine owing to their self-renewal ability and multiple differentiation potential. However, the serious shortage of MSCs and the difficulty of maintaining the differentiation potential during culture *in vitro* limit their application. Microcarrier culture technology is an effective method to realize large-scale culture of MSCs, whereas the effect of microcarriers properties on MSCs proliferation and differentiation potential maintenance should be investigated. In this study, Konjac glucomannan (KGM) microcarriers with a wide range of rigidity from 0.2 MPa to 10 MPa were prepared using water-in-oil emulsion polymerization. It was found that the microcarriers rigidity had great influence on MSCs by regulating cell spreading rate. Rapid proliferation and good differentiation potential maintenance of MSCs were achieved on the intermediate rigidity about 2.51 ± 0.65 MPa with the faster spreading rate of cells. As a result, MSCs on 2.51 ± 0.65 MPa KGM microcarriers proliferated about 27 times, which was 1.7 times as much as MSCs cultured on the commercial microcarriers Cytodex-1. The differentiation potential was also improved about 3.2 times compared to Cytodex-1. Therefore, it is indispensable to regulate microcarriers rigidity, especially for application of MSCs.

Keywords MSCs; Proliferation; Differentiation potential; Rigidity; Spreading

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INTRODUCTION

Mesenchymal stem cells (MSCs) are a kind of multipotent stem cells with self-renewal ability and multilineage differentiation potential. They can be isolated from various tissues such as the bone marrow, adipose tissue, dental pulp, synovial membrane, as well as from perinatal sources such as the umbilical cord matrix, umbilical cord blood, amniotic fluid and the placenta.^[1,2] In addition, they can differentiate into various tissue cells such as neurocytes, myocardial cells, osteoblasts, chondrocytes and adipocytes. Owing to their availability from a wide range of sources and multilineage differentiation ability, MSCs are seen as a promising candidate in cell-based therapies for different diseases^[3] including myocardial infarction, graft versus host disease, Crohn's disease, acute lung injury, bone regeneration, cartilage regeneration, and multiple MSCs products have been clinically approved nowadays.^[4] Compared to the increasing demand and a huge number of MSCs needed for clinical therapies, such as 100–400 million per patient for graft versus host disease and 3000–6000 million per patient for osteogenesis imperfecta.^[5] But, the isolation efficiency is rather

limited. For instance, MSCs contain only 0.001%–0.01% of the total mononuclear cells in the bone marrow, that is, about 0.2–2 million MSCs obtained from 100 mL bone marrow aspirate.^[6,7] Thus, large-scale cultural technology is a prerequisite for the biomedical applications of MSC-based therapies. On the other hand, the proliferation rate of MSCs is fast when it maintains stemness, while it slows down once the cells are differentiated or aging.^[8] However, it is difficult to maintain their stemness during their culture *in vitro* because they are highly susceptible to the biochemical and biophysical properties of the environment.^[9] Therefore, the properties of the culture system are important to manipulate for MSCs rapid proliferation and stemness maintenance.

Microcarrier cell culture technology is a promising technique that combines the advantages of monolayer adherent culture and suspension culture. The surface of microcarriers is available for cell adhesion and growth, while the suspension of microcarriers in the medium generates a homogeneous culture environment. Compared to traditional 2D culture flasks, microcarriers provide a high surface area to volume ratio due to the small particle size (about 200 μm). Take the commercial microcarriers Cytodex-1 as an example, 3 g Cytodex-1 microcarriers in a 1 L reactor can supply a surface area of 1.32×10^4 cm^2 which is equivalent to 176 culture flasks with 75 cm^2 each, leading to a great reduction in laboratory space and the number of operating steps, thus dramatically reducing labor costs and risks of contamination. Furthermore, a

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homogeneously-stirred microcarriers suspension culture allows real-time monitoring and controlling of cell culture process and various environmental parameters such as pH, O₂ and the concentration of medium components.^[5] Therefore, microcarriers technology has great advantages in the large-scale culture of MSCs.

As cell-matrix interactions are central to stem cells behaviours, many materials with different chemical and physical properties have been designed to manipulate cell fate.^[9,10] Based on the studies in 2D culture, materials regulate cells behaviours through cell adhesions,^[8,11] which are stimulated by the binding of the transmembrane proteins called integrins and their ligands on materials. The linkage of integrins and their ligands triggers the recruitment and activation of adhesion proteins such as vinculin and talin, which link the cytoskeleton and materials and serve as the structure of force transmission.^[12,13] In addition, Tyr kinase focal adhesion kinase (FAK) is also recruited, which regulate Rho GTPase related to actin polymerization, spreading and cell contraction force, and ERK1/2 involve in cell proliferation and differentiation.^[14] Therefore, the extent of adhesions controls the level of these proteins recruitment and activation, and thus different cell behaviours. For example, MSCs that can attach, but that cannot form mature adhesions, differentiate into adipocytes, while MSCs which form large, and supermature adhesions differentiate into osteoblasts. Although some researchers suggest that an intermediate cell adhesion state is required to prevent MSCs differentiation and enable long-term MSCs growth *in vitro*,^[8] the exactly quantitation standard of adhesions remains unclear. Materials rigidity is an important parameter that regulates cell adhesions through cytoskeleton contractions.^[15] Stiff matrix triggers larger cytoskeleton contraction force and as a result of larger and more stable adhesions than soft matrix. However, how much rigidity of matrix stimulates the intermediate adhesion for MSCs stemness maintenance requires more researches. Therefore, in order to develop the specific microcarriers for the rapid proliferation and stemness maintenance of MSCs, it is necessary to explore the effect of rigidity of microcarriers on MSCs proliferation and differentiation potential.

Konjac glucomannan (KGM) microspheres with good stiffness controllability were prepared in order to investigate the above-mentioned problem. KGM is a kind of natural water-soluble polysaccharide with favorable biocompatibility from the high-yield economic plant *Amorphophallus Konjac*.^[16] KGM contains a backbone chain of β -1,4 linked D-mannose and D-glucose with a low degree of acetyl groups.^[17] Owing to the molecules rich of active groups like hydroxy, KGM is easy to be crosslinked and modified by series of ligands such as DEAE, collagen and peptide for cell adhesion.^[18–20]

In this study, we prepared KGM microcarriers using water-in-oil emulsion polymerization and the rigidity was controlled by modulating KGM concentration in the aqueous phase. The microcarriers were aminated with DEAE to carry positive charge for cell adhesion and microcarriers with anion exchange capacity (AEC) from 0.8 mmol/g to 1.8 mmol/g dry weight were prepared to obtain the appropriate charge density for MSCs adhesion and proliferation. In order to get the optimal rigidity for MSCs rapid proliferation and good tri-lineage differentiation potential, we measured the cell num-

ber, detected the surface marker expression levels and the tri-lineage differentiation potential of MSCs after the cell number reach the peak. In addition, we compared our KGM microcarriers with optimized rigidity to the commercial products Cytodex-1 as well.

EXPERIMENTAL

Materials

The powder of KGM was purchased from Hubei Konson Konjac Gum Co., Ltd. (Hubei, China). NaOH, HCl, NaCl were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). DEAE-HCl was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Cytodex-1 microcarriers were purchased from GE Healthcare (Uppsala, Sweden). hBM-MSCs were provided from BeNa Culture Collection. (Beijing, China). High-glucose Dulbecco's modified eagle's medium (DMEM) were from HyClone (USA). Fetal bovine serum (FBS), 0.25% trypsin-EDTA, and penicillin-streptomycin were supplied from Gibco (USA). Glutamine was purchased from Beyotime (Shanghai, China). Triton X-100, bovine serum albumin (BSA), 4% paraformaldehyde, DAPI, and the powder of crystal violet were purchased from Solarbio (Beijing, China). Antibodies for CD105, CD90, CD73, CD34, CD45, CD14, CD19, HLA-DR were purchased from eBioscience (USA). Antibody for GAPDH was purchased from Affinity (USA). Rhodamine-phalloidin, antibodies for AP2, collagen II, osteocalcin (OCN) and horseradish peroxidase conjugated anti-rabbit IgG were purchased from Abcam (UK). Human MSC Adipogenic, Chondrogenic, and Osteogenic Differentiation Medium were provided by Cyagen Biosciences Inc. (Guangzhou, China). All human stem cells experiments were proved by local Expert Committee on Biomedical Ethics, complying with the "Guidelines for Stem Cell Research and Clinical Translation" published by International Society for Stem Cell Research (ISSCR) (Version 1.0, May 2021).

Preparation of the KGM Microcarriers

KGM microspheres were prepared by using water-in-oil (W/O) emulsion as described previously.^[21] The formed microspheres contained 8%, 10%, 12% and 16% KGM and 25% crosslinker. Microspheres with diameters between 150 and 200 μ m were sieved out and used for further modification. Amination process was as the following: 10 g of vacuum-dried KGM microspheres were mixed with 5 g of 14 g/mL NaOH solution and stirred at 70 °C with 120 r/min for 1 h. Then DEAE-HCl was added at the specific ratio to microspheres weight to form KGM microcarriers with anion exchange capacity (AEC) of 0.8, 1.3, 1.6 and 1.8 mmol/g dry weight. The reaction mixture was stirred for 4 h, and the formed KGM microcarriers were washed with deionized water, incubated with HCl solution (pH 2) overnight, and finally washed with deionized water until the pH value of the eluate achieved neutral.

Characterization of KGM Microcarriers Structure

The surface morphology of the KGM microcarriers was observed by an optical microscope and a scanning electron microscope (SEM). For SEM observation, dry KGM microcarriers and Cytodex-1 were coated with platinum under vacuum by an ion sputter (JFC-1600, JEOL) and observed using a JEM-6700F SEM (JEOL, Japan).

Measurements of KGM Microcarriers Anion Exchange Capacity (AEC)

The DEAE substitution degree, in other words, anion exchange capacity of the KGM microcarriers was measured by the method described previously.^[18] Vacuum-dried KGM microcarriers (5 g) were further dried in an oven at 105 °C until no weight loss and we got the dry weight of KGM microcarriers (m_1). At the same time, another vacuum-dried KGM microcarriers (5 g) were soaked in deionized water and packed into a test column. Firstly, 50 mL of 0.5 mmol/mL NaOH solution passed through the column at 0.8–1.0 mL/min. Then, in order to remove residual NaOH that was not reacted, the column was washed with distilled water until the phenolphthalein-containing eluate displayed no color. After that, 25 mL (V_1) of standard HCl solution (0.1 mmol/mL (C_1)) flowed through the column at the rate of 0.4–0.5 mL/min, 1 mmol/mL NaCl was then used for washing the column to wash the unreactive HCl. The eluates were pooled, and 2–3 drops of phenolphthalein were added, and then this mixture was titrated with 0.1 mmol/mL (C_2) standard NaOH until the solution displayed reddish. The volume of NaOH used was defined as V_2 . The anion exchange capacity (AEC) of the KGM microcarriers was calculated according to the following formulation:

$$\text{AEC (mmol/g dry weight)} = \frac{(C_1 V_1 - C_2 V_2)}{m_1} \quad (1)$$

Measurements of KGM Microcarriers Rigidity

The rigidity (Elastic modulus) of KGM microcarriers was measured by atomic force microscopy (AFM) (Bruker) in the model of “contact in fluid” which aimed to avoid stiffening of microcarriers in the state of dehydration. Briefly, KGM microcarriers were first dehydrated using graded ethanol and dried naturally in order to ensure the microcarriers can be attached to the glue, which can't be attached in the state of hydration. Dry samples were fixed on waterproof glue evenly for 1 h and then rehydrated for above 4 h. Pyramidal tips which was special for the model of “contact in fluid” with an effective half-angle α of 20° and a nominal cantilever's spring constant of $K=0.4\text{--}0.6$ N/m were used. The actual spring constant was calibrated by thermal tuning using the simple harmonic oscillator model. For each rigidity, ten or more microcarriers were measured. To compute the elastic modulus (E), we fitted the Sneddon model equation as shown below for pyramidal tips to the force-displacement curves:

$$F(\delta) = \frac{2}{\pi} \cdot E \cdot \tan \alpha \cdot \delta^2 \quad (2)$$

$$F(\delta) = Kd \quad (3)$$

$$\delta = \Delta z - d \quad (4)$$

where α is the half-angle of the indenter, δ is indentation depth, Δz is the cantilever's vertical displacement and d is its deflection, K is the cantilever's spring constant.

MSCs Culture and Cell Density Measurements on KGM Microcarriers

KGM microcarriers were suspended in PBS and sterilized by autoclaving at 121 °C for 30 min and then stored at 4 °C. Before use, the microcarriers were settled, washed twice and equilibrated with complete medium (high-glucose Dulbecco's modified eagle's medium (DMEM, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA),

2.0 mmol/L glutamine (Beyotime, China), and 100 U/mL penicillin-streptomycin (Gibco, USA)) for at least 2 h at 37 °C. Prior to each experiment, human bone marrow mesenchymal stem cells (hBM-MSCs) were cultured in 75 cm² flasks to 80%–90% confluence, and then were detached with 0.25% trypsin-EDTA (Gibco, USA). The cell density was measured by the automatic cell counter (Invitrogen, USA). The KGM microcarriers with a final concentration of 2.0 mg/mL and MSCs with a final concentration of 1.0×10^5 cells/mL were added successively to the six-well cell culture plate with a low-attachment surface. Partial medium exchange was conducted every day. Samples were collected to monitor the cell growth. Cell morphology was observed using an inverted microscope (Optec, China). Cell density was counted with crystal violet nuclei-staining method. Briefly, 0.1 mL of well-mixed sample was washed twice with PBS, then treated with 0.1 mL of 0.1 mol/L citric acid containing 0.1% crystal violet and incubated for 1 h at 37 °C. The released nuclei were counted using a haemocytometer.

Immunostaining and Cell Area Measurements

MSCs cultured on KGM microcarriers with different rigidity were washed with PBS for 3 times, fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 (Solarbio, China) for 5 min and blocked with 3% BSA (Solarbio, China) for 1 h, following which cells were incubated with rhodamine-phalloidin (Abcam, UK) for 90 min at room temperature. Nuclei were labelled by DAPI (Solarbio, China) before being imaged. 3D confocal imaging was acquired with a $\times 60$ oil-immersion objective using a CLSM (Nikon A1R, Japan). To quantify cell area, we identified cells manually and measured their area.

Cell Surface Markers Examination

The specific cell surface markers of hBM-MSCs were examined *via* flow cytometry (Beckman Coulter). The antibodies for positive markers included CD105, CD90 and CD73, whereas the negative markers included CD34, CD19, CD14, CD45 and HLA-DR (eBioscience, USA).

Trilineage-induced Differentiation Experiment

A trilineage-induced differentiation experiment was also performed to identify the multiple differentiation potential of hBM-MSCs, which included adipogenesis, osteogenesis, and chondrogenesis.

Briefly, the hBM-MSCs cultured on KGM microcarriers for 11 days were digested and seeded in the six-well plate at a density of 1×10^5 cells per well with Human MSC Adipogenic or Osteogenic Differentiation Medium (Cyagen Biosciences Inc, China) for adipogenesis or osteogenesis induction, respectively. For chondrogenesis, pellet culture was performed. Briefly, the hBM-MSCs were digested and resuspended in DMEM in a 15 mL of polypropylene centrifuge tube. A total of 1×10^6 cells/tube were washed with DMEM twice at 1000 r/min for 5 min, resuspended in 0.5 mL of Human MSC Chondrogenic Differentiation Medium (Cyagen Biosciences Inc, China), and centrifuged at 1000 r/min for 5 min. The pellet was incubated at the bottom of the tube with the supernatant at 37 °C in 5% CO₂ for 24 h, and the tube was gently flicked to ensure the pellet was free-floating. The medium was changed every 2–3 days.

Histological Staining and Western Blotting

For differentiation examination, qualitative detection with

histological staining and quantitative detection with western blotting were performed. The cells were examined for adipogenesis through oil red O staining after two weeks of culture, or for osteogenesis through alizarin red staining after two weeks of culture. For chondrogenesis, after five weeks of incubation, the pellet was fixed in 4% paraformaldehyde (Solarbio, China), and embedded in paraffin for toluidine blue staining. After induction, total proteins were extracted by cellular lysates and were separated by SDS-PAGE. Primary antibodies for GAPDH (1:1,5000, Affinity, USA), AP2 (1:500, Abcam, USA), collagen II (1:1000, Abcam, UK), OCN (1:500, Abcam, UK) and horseradish peroxidase conjugated anti-rabbit IgG (1:8000, Abcam, UK) were used for immunoblotting analysis.

Statistical Analysis

ANOVA was performed to analyze the results and P value of less than 0.05 was considered to indicate a statistically significant difference. Data were reported as means $\pm$ standard deviation of duplicated experiments.

RESULTS AND DISCUSSION

Rigidity Modulation and Characteristics of KGM Microcarriers

KGM microcarriers were prepared by water-in-oil emulsion polymerization and aminated with DEAE for cell adhesion and growth. Controlling the KGM concentration in the aqueous phase can modulate the skeleton density of the microcarriers, and thus the rigidity. We prepared the microcarriers with KGM concentrations of 8%, 10%, 12% and 16%. KGM microcarriers

were named as 8%-KGM, 10%-KGM, 12%-KGM, 16%-KGM microcarriers.

As shown in Fig. 1(a), all KGM microcarriers were spherical, transparent and the surface was smooth without nanostructure, indicating that the effect of microcarriers with different KGM concentrations on MSCs was caused only by rigidity. The rigidity was characterized by elastic modulus which was strain under stress. According to Fig. 1(b), the rigidity of KGM microcarriers with different KGM concentrations of 8%, 10%, 12%, 16% were 0.18 ± 0.03 MPa, 0.96 ± 0.12 MPa, 2.51 ± 0.65 MPa and 9.73 ± 1.68 MPa, respectively. We also characterized the surface structure and rigidity of the commercial microcarriers Cytodex-1 and found that Cytodex-1 was not smooth but a cristae-like micro-rough structure and the rigidity was about 0.87 ± 0.78 MPa which was similar to 10%-KGM microcarriers.

Effect of KGM Microcarriers Anion Exchange Capacity on MSCs Adhesion and Proliferation

To investigate the appropriate anion exchange capacity for MSCs adhesion and proliferation, DEAE aminated KGM microspheres with the anion exchange capacity from 0.8 mmol/g to 1.8 mmol/g dry weight were prepared. Experimental results showed that cells can attach on all KGM microcarriers after culture for 24 h, but when the anion exchange capacity was low, they cannot distribute evenly and large numbers of cells did not spread and grew as cell spheroids (Fig. 2a) and also proliferated slowly (Fig. 2b). When the anion exchange capacity increased to 1.3 mmol/g, cells can distribute more uniformly and proliferate faster, until to 1.8 mmol/g, the proliferation rate no longer increased. It has been well known that cells sense the

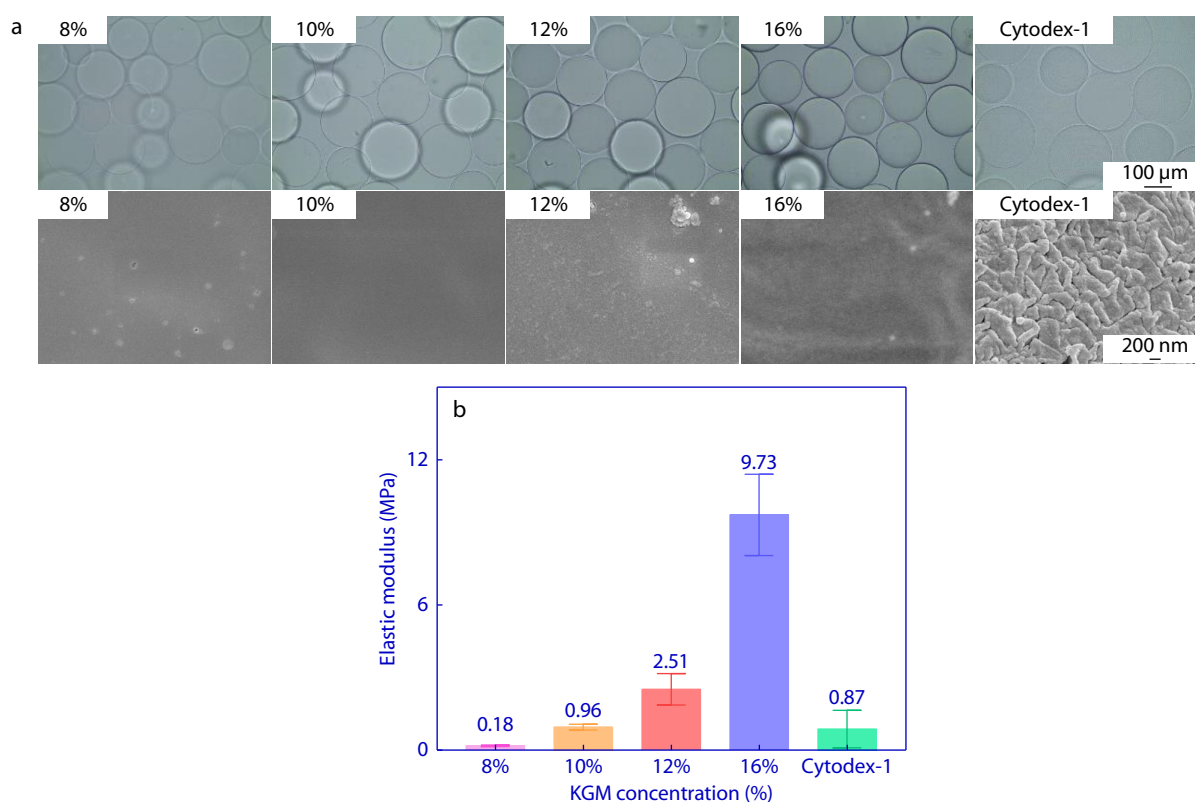


Fig. 1 Characteristics of the KGM microcarriers and Cytodex-1 structure and rigidity. (a) Optical microscope (scale bar: 100 μ m) and SEM images (scale bar: 200 nm) of the microcarriers. (b) The rigidity of the Cytodex-1 and KGM microcarriers, with different concentration of KGM.

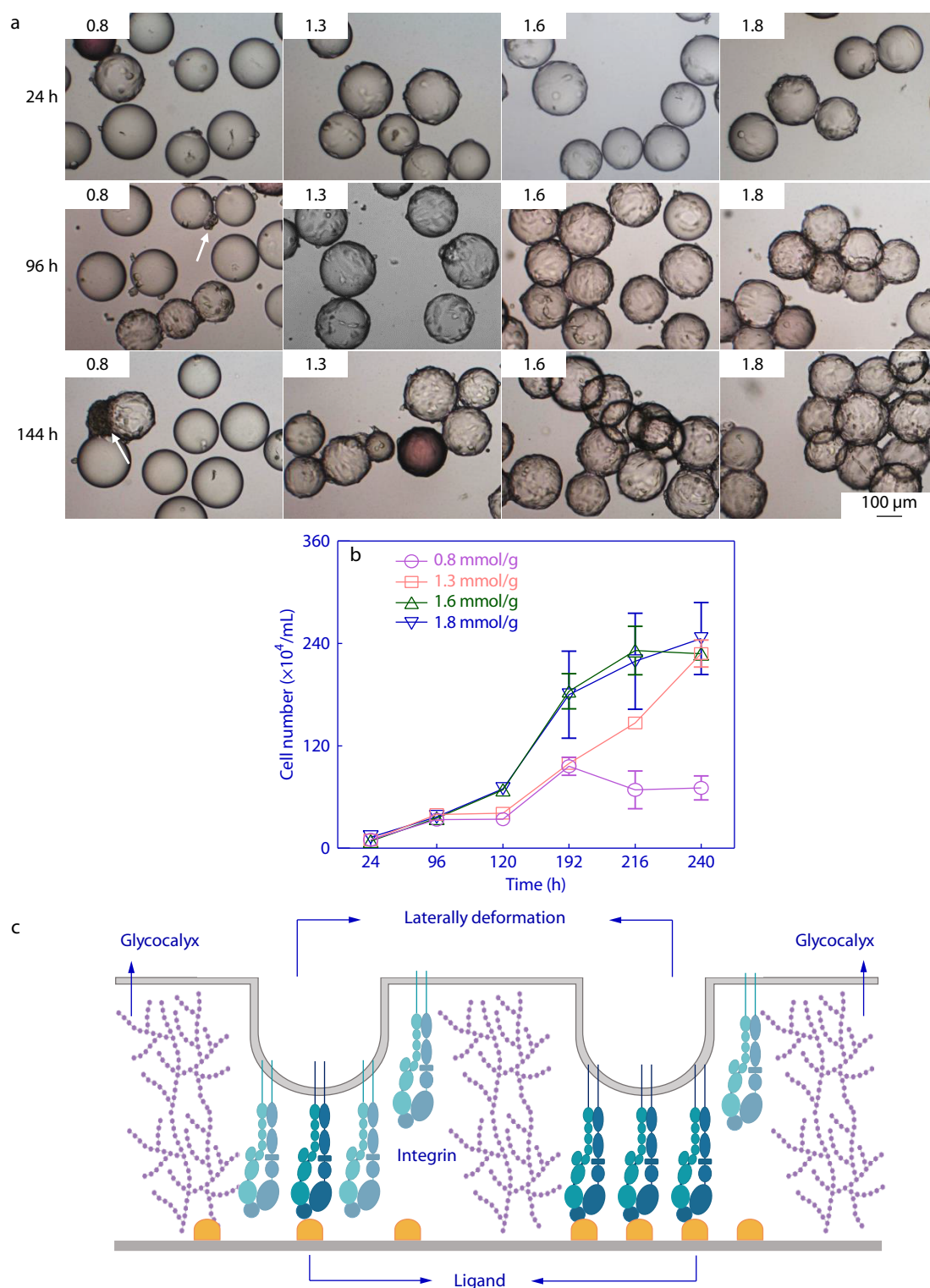


Fig. 2 Effect of KGM microcarriers anion exchange capacity on MSCs adhesion and proliferation. (a) Inverted microscope images of cells on 12%-KGM microcarriers with different AEC at 24 h, 96 h, 144 h. Scale bar: 100 μm. (b) Cell growth curve of MSCs on 12%-KGM microcarriers with different anion exchange capacity. (c) Model of effect of ligand distance on the integrin clustering and adhesions formation.

environment through their transmembrane receptors such as integrins and a limited distance between ligands which integrins bind to is required for cell spreading and mature adhesion formation, whereas above this distance, integrins

clustering and maturation of adhesion are inhibited.^[22–24] The reason why the maximum distance between two ligands is required may be related to the process of integrin clustering. Integrin-ligand bond formation occurs when the two molecules

are close to each other, however, glycocalyx on surface of cell membrane extends beyond about the 20-nm length of the integrin, which serves as a steric barrier impairing integrin-ligand binding. To bridge this physical glycocalyx barrier and bind their ligand, integrins need to locally bend the membrane towards the ligand, which causes a highly localized deformation that extended laterally in the plane of the membrane with a certain width from the bound site. When the distance between two ligands less than the laterally width, the adjacent integrin-ligand bond formation, integrin clustering occurs, while when the distance between two ligands is above the laterally width, the adjacent integrin is inaccessible to its ligand, integrin clustering is inhibited (Fig. 2c).^[25] With the decrease of distances between two bound integrins, larger adhesion forms and cells spread larger.^[26] However, a minimum distance also exists owing to the restriction of the physical size of adhesions, stress fibers, and cells themselves.^[27] Furthermore, the spreading area and adhesion size were related to cell proliferation.^[15] In our study, the anion exchange capacity reflects charge density and thus the distance between two adhesion points binding integrins. Therefore, when the anion exchange capacity was as low as 0.8 mmol/g, the distance between adhesion points may be larger than the optimized distance so that inhibited cell spreading. With the increase of the anion exchange capacity to 1.3 mmol/g, the distance between adhesion points become smaller and closer to the optimized distance, which promotes cell spreading. When the anion exchange capacity increased from 1.3 mmol/g to 1.6 mmol/g, cell proliferated faster probably because of the increasing adhesion or spreading area. There was no difference between 1.6 mmol/g and 1.8 mmol/g, so they provided an appropriate interforce between cell and microcarrier. And then 1.8 mmol/g was used for the subsequent experiments.

Effect of KGM Microcarriers Rigidity on MSCs Proliferation and Differentiation Potential

To investigate the effect of KGM microcarriers rigidity on MSCs proliferation, MSCs were cultured on KGM microcarriers with different rigidity and similar anion exchange capacity (1.8 mmol/g). According to the cell growth curve (Fig. 3a) and the histogram of the proliferation fold (Fig. 3b), we found that KGM microcarriers rigidity has a prominent effect on the proliferation of MSCs. From 8%-KGM to 12%-KGM microcarriers, the higher

KGM microcarriers rigidity was, the faster MSCs proliferated. The proliferation rate reached the maximum when MSCs grown on 12%-KGM microcarriers with a rigidity of 2.51 ± 0.65 MPa, and then decreased as the rigidity was further increased, indicating that the optimal rigidity of KGM microcarriers for MSCs was 2.51 ± 0.65 MPa. After 11 days of culture, MSCs on 12%-KGM microcarriers proliferated 27 times.

In addition to the rapid proliferation rate, the maintenance of the cell properties was also necessary. According to the International Society for Cellular Therapy (ISCT), MSCs were defined with three criteria: First, MSCs must be plastic-adherent. Second, above 95% of the MSCs population must express CD105, CD73, CD90, and these cells must lack expression (below 2% positive) of CD45, CD34, CD14, CD19 and HLA-DR. Third, the cells must have trilineage differentiation potential to osteoblasts, adipocytes and chondroblasts.^[28] The flow cytometry results showed that MSCs on all KGM microcarriers maintained their specific phenotype positive for CD105, CD90, CD73 (>95%) and negative for CD45, CD34, CD14, CD19, HLA-DR (<2%), indicating that KGM microcarriers rigidity can't affect MSCs purity (Fig. 4).

To investigate the effect of microcarriers rigidity on trilineage differentiation potential, MSCs cultured for 11 days on KGM microcarriers were digested, seeded on cell culture plates, and were cultured in specific lineage induction media. As shown in histological staining results (Fig. 5a), MSCs on all KGM microcarriers had trilineage differentiation potential, but there were significant differences among the different microcarriers according to western blotting results (Fig. 5b). The osteogenic and adipogenic differentiation potential had no significant difference among these KGM microcarriers, while MSCs exhibited the highest cartilage differentiation potential on 12%-KGM microcarriers, on which MSCs also had the fastest proliferation rate. Therefore, the KGM microcarriers with a rigidity of 2.51 ± 0.65 MPa facilitated MSCs rapid proliferation and good stemness maintenance.

Effect of KGM Microcarriers Rigidity on MSCs Spreading Rate

According to the confocal images (Fig. 6), we noticed that MSCs on KGM microcarriers with different rigidity spread with different rate. After cultured for 24 h, the spreading area of MSCs

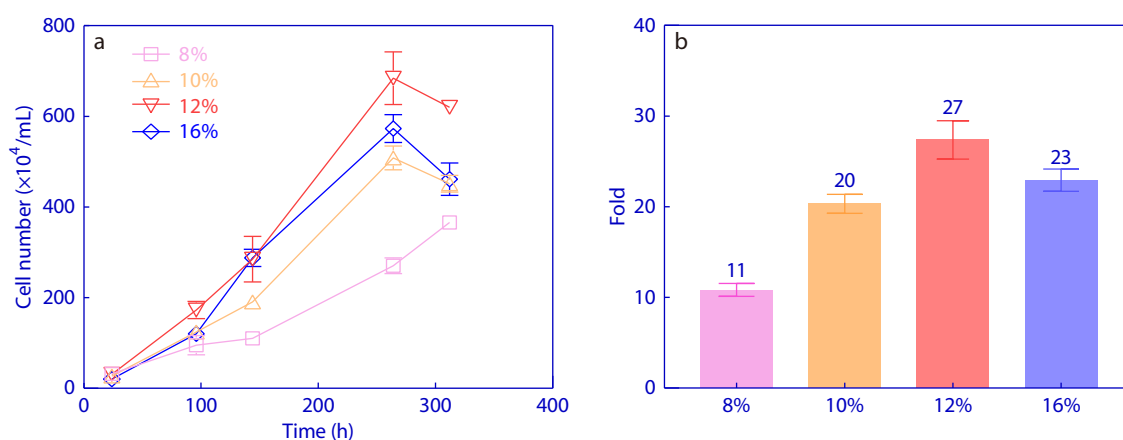


Fig. 3 Effect of KGM microcarriers rigidity on MSCs proliferation. (a) Cell growth curve of MSCs on KGM microcarriers with different rigidity. (b) Proliferation fold of MSCs on different KGM microcarriers.

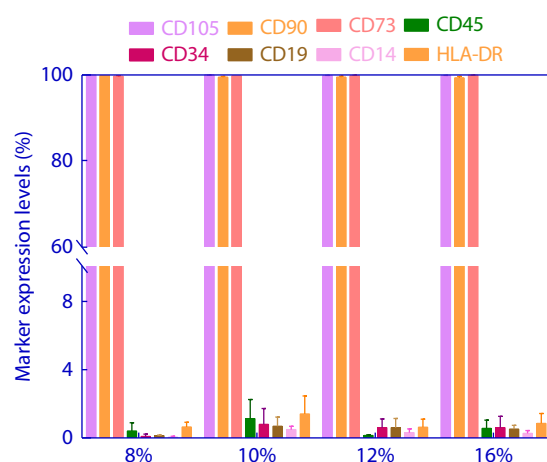


Fig. 4 Effect of KGM microcarriers rigidity on MSCs surface marker expression. Specific cell surface markers of MSCs were tested *via* flow cytometry analysis.

increased first and then decreased with the increase of microcarriers rigidity, which was consistent with the rule of cell proliferation and differentiation potential. Cell spreading is a process involved in actin polymerization and adhesion formation, which stimulate various signaling proteins related to proliferation and cell fate. Actin polymerization makes cell spread forward, and the adhesions stabilize the spreading by linking the actin cytoskeleton to the underlying matrix.^[29] Therefore, the spreading rate depends on the rate of actin polymerization minus the rate of actin retrograde flow caused by membrane tension and actomyosin contraction. The actin polymerization is regulated by Rho GTPase containing Rac and RhoA,^[30] which is stimulated by Tyr kinase focal adhesion kinase (FAK) recruited to adhesions. Adhesions function as traction points that resist the force arising from the rearward flow of actin filaments. Therefore, the actin retrograde flow slows down when the adhesions are matured and stabilized by the force of actomyosin contraction.^[31,32] According to these, cell spreading rate may be consistent with the adhesion size and stability

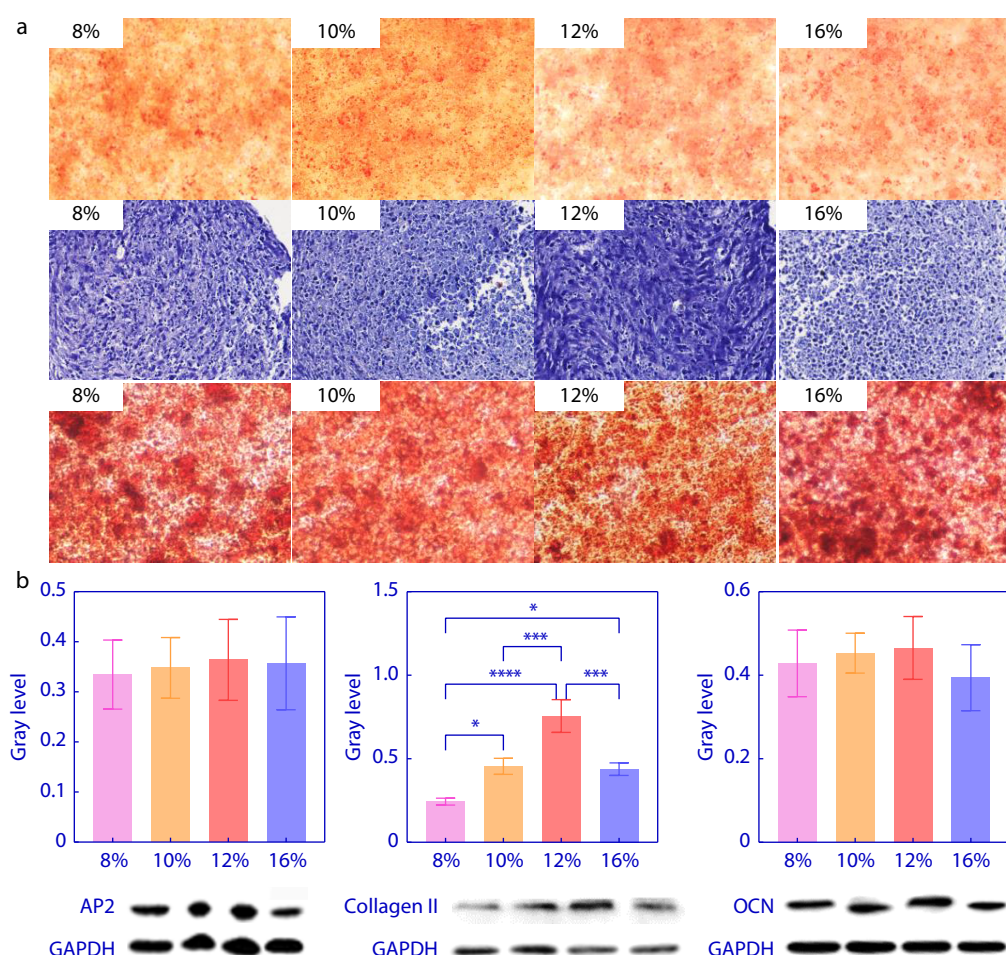


Fig. 5 Effect of KGM microcarriers rigidity on MSCs differentiation potential. (a) Histological staining of MSCs from different KGM microcarriers induced by specific lineage media. The adipogenesis potential was checked through oil red O staining (the first row), and the chondrogenesis potential was assessed by toluidine blue staining (the second row) and the osteogenesis potential was examined using alizarin red staining (the third row). (b) Western blotting analysis of MSCs from different KGM microcarriers induced by specific lineage media. The adipogenesis potential was checked through AP2 (the first column), the chondrogenesis potential was assessed by collagen II (the second column) and the osteogenesis potential was examined using OCN (the third column).

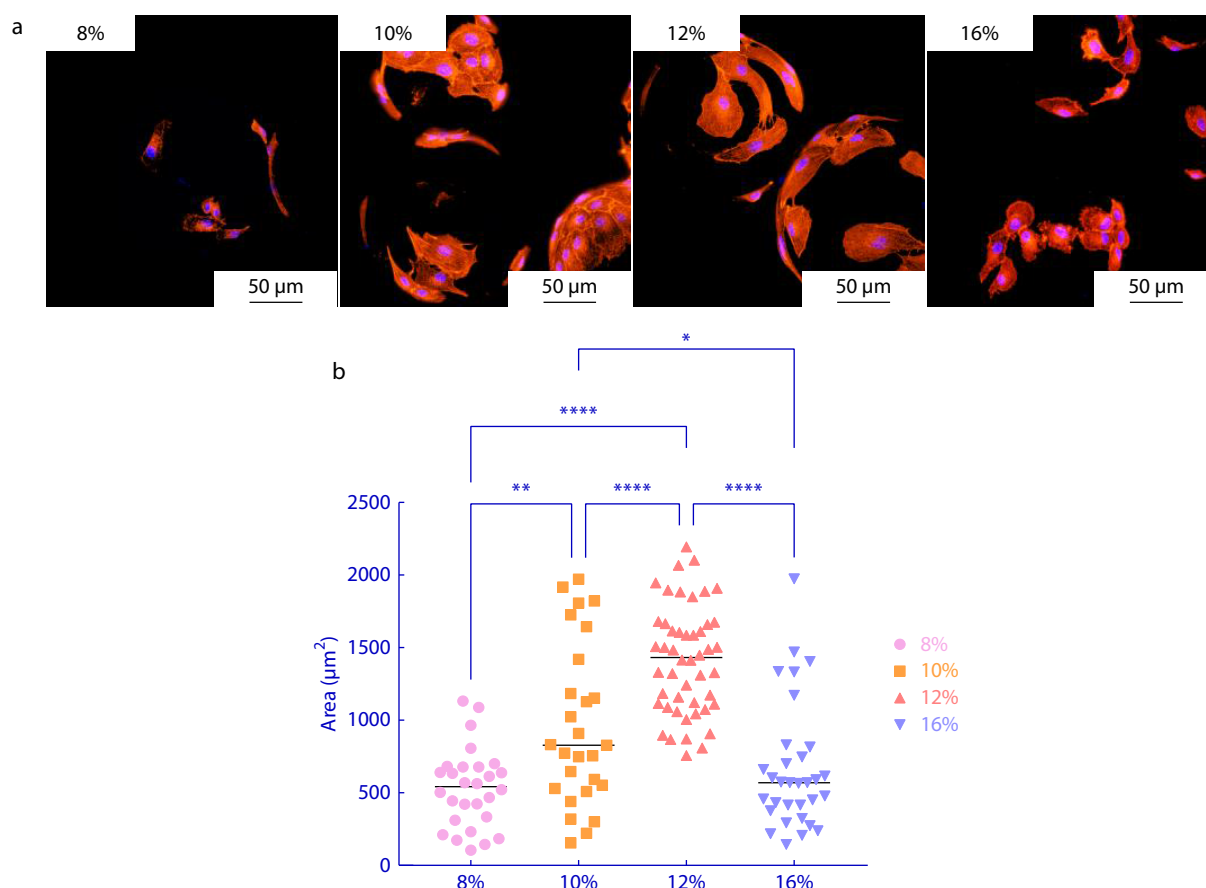


Fig. 6 Effect of KGM microcarriers rigidity on MSCs spreading. (a) Cytoskeleton and nucleus were imaged by rhodamine phalloidin (red) and DAPI (blue). Scale bar: 50 μm. (b) Spreading area of MSCs on different KGM microcarriers after cultured for 24 h were measured.

related to the force of actomyosin contraction, while larger and more stable adhesion indicates more proteins expression such as FAK, which regulated mitogen-activated protein kinase (MAPK) extracellular signal-related kinase (ERK) 1/2 that related to cell proliferation and fate.^[8] In addition, it has been well known that the force of actomyosin contraction depend on matrix rigidity, and stiff or soft matrix leading to higher or lower forces, respectively.^[33] Therefore, in general speaking, stiffer matrix triggers larger and more stable adhesions and thus faster spreading rates.^[15] In our study, the rule of spreading rate from 8%-KGM to 12%-KGM microcarriers was consistent with the common sense, while it was interesting that the cell spreading rate decreased with the rigidity increased further (16%-KGM microcarriers). This was probably because the actomyosin contraction force of MSCs on this microcarrier was too larger which suppressed the threshold that the adhesions can stand and led to the collapse of adhesions,^[26] resulting in the higher rate of retrograde flow and slower rate of cell spreading. Therefore, we speculated that it was rigidity of microcarriers regulated the proliferation and differentiation potential by controlling the adhesion formation and cell spreading rate in a mechanical manner.

Comparison of KGM Microcarriers and Commercial Microcarriers Cytodex-1

We compared the KGM microcarriers with the optimal rigidity with the commercial microcarriers Cytodex-1. As shown in Fig. 7,

the proliferation rate of MSCs on 12%-KGM microcarriers was significantly higher than that on Cytodex-1 and the maximum cell number was 1.7 times as much as that on Cytodex-1. In addition, as for trilineage differentiation potential (Fig. 8), the cartilage differentiation potential was about 3.2 times as much as MSCs on Cytodex-1. The reason probably was that the rigidity of Cytodex-1 was 0.87 ± 0.78 MPa, which was not the optimal rigidity for MSCs proliferation and differentiation potential maintenance. Even though it has been reported that the cristae-like micro-rough surface structure facilitates cells adhesion and growth,^[34,35] it cannot eliminate the negative effect of improper rigidity. Therefore, the 12%-KGM microcarriers with 2.51 ± 0.65 MPa was more promising for MSCs large-scale culture.

CONCLUSIONS

In this study, we prepared the KGM microcarriers with an appropriate charge density and explored the effect of the microcarriers rigidity on MSCs spreading, proliferation and differentiation potential. The results showed that MSCs proliferation rate and differentiation potential increased with the increase of the KGM microcarriers rigidity and reached the maximum when the rigidity improved to 2.51 ± 0.65 MPa. With the further increase of the rigidity, MSCs proliferation rate and differentiation potential decreased. The cell spreading rate and area had the similar rule. Considered that the cell spreading rate was related to the adhesion maturity and stability which

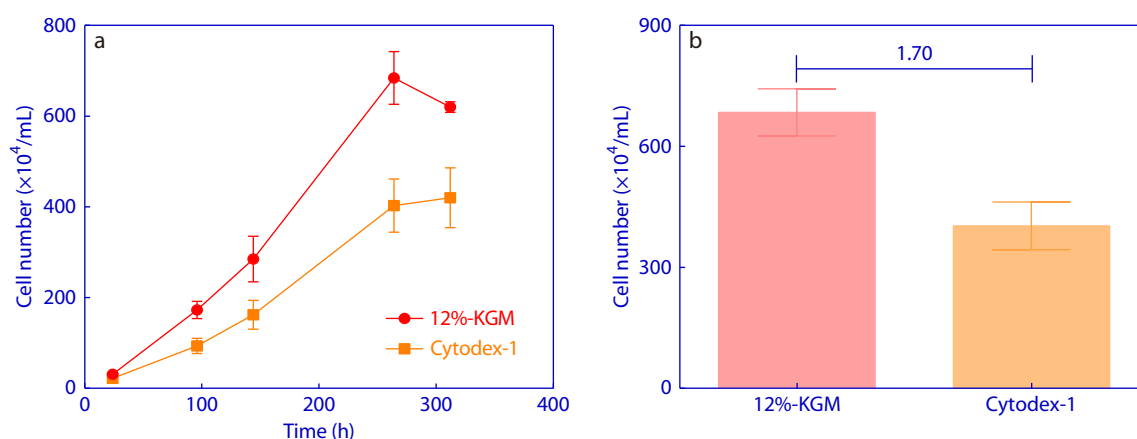


Fig. 7 Comparison of cell proliferation between 12%-KGM microcarriers and commercial microcarriers Cytodex-1. (a) Cell growth curve of MSCs on 12%-KGM microcarriers and Cytodex-1; (b) Maximum cell numbers of MSCs on 12%-KGM microcarriers and Cytodex-1.

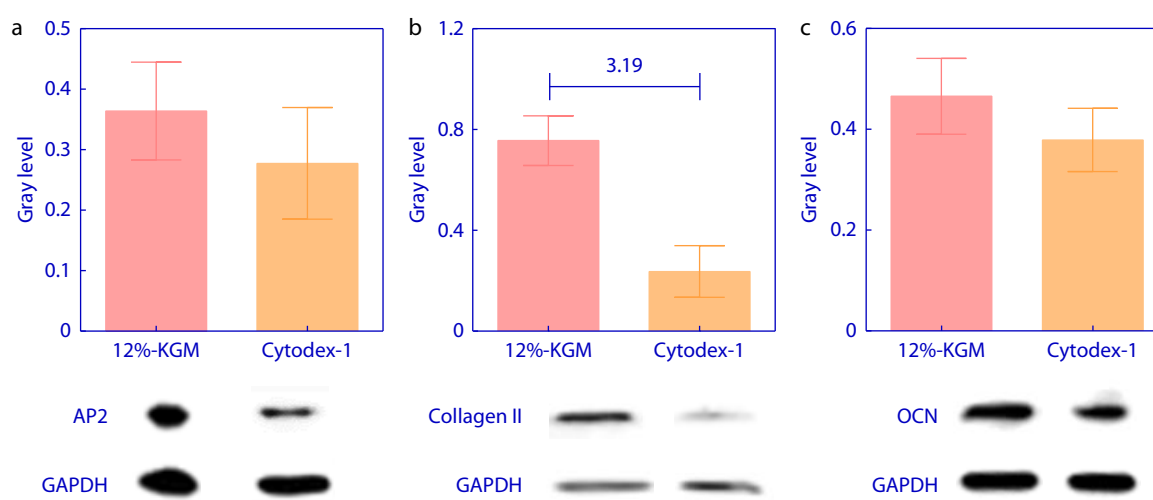


Fig. 8 Comparison of trilineage differentiation potential between 12%-KGM microcarriers and commercial microcarriers Cytodex-1. Western blotting analysis of MSCs from 12%-KGM microcarriers and Cytodex-1 induced by specific lineage media. The adipogenesis potential was checked through AP2 (a), the chondrogenesis potential was assessed by collagen II (b), and the osteogenesis potential was examined using OCN (c).

regulated by the force of actomyosin contraction, we speculated that the rigidity of microcarriers regulated the proliferation and differentiation potentials by regulating the cell spreading in a mechanical manner. With the optimal KGM microcarriers rigidity, MSCs proliferated about 27 times after 11 days, which was 1.7 times as much as commercial product Cytodex-1, and the differentiation potential was 3.2 times as much as Cytodex-1. These results show that MSCs are sensitive to the rigidity of microcarriers and the KGM microcarriers with optimized rigidity provide a promising select for MSCs large-scale culture and better application. Furthermore, the KGM microcarriers with controllable rigidity probably can be used for other stem cells as well, even though the optimal rigidity would be different.

NOTES

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