Biomaterials 34 (2013) 4725–4738

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

# Substrate-dependent Wnt signaling in MSC differentiation within biomaterial-derived 3D spheroids

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ARTICLE INFO

*Article history:*
Received 25 February 2013
Accepted 11 March 2013
Available online 2 April 2013

*Keywords:*
Mesenchymal stem cells (MSCs)
Spheroids
Wnt signaling
Chitosan
Hyaluronan

ABSTRACT

A unique biomaterial-based system was developed to generate dynamic three-dimensional (3D) multicellular spheroids of mesenchymal stem cells (MSCs). MSCs were cultured on transparent membranes made of chitosan or those further grafted with hyaluronan (HA) in different densities. MSCs vigorously migrated and were self-assembled into highly mobile 3D spheroids with substrate-dependent upregulation of adhesion molecule N-cadherin. MSC spheroids showed increased expression of Wnt genes/proteins and substrate-dependent cell fate. The correlation of differentiation capacities with Wnt signaling and crosstalk with other pathways such as ERK1/2 or Smad2/3 were observed for MSC spheroids but not for the conventional 2D cultured cells. Wnt3a-mediated canonical Wnt signaling was more active for MSC spheroids derived on chitosan, which were prone to osteogenesis. Wnt5a-mediated non-canonical Wnt signaling was more active for MSC spheroids derived on HA-grafted chitosan, which were prone to chondrogenesis. In particular, the relative importance of Wnt5a-mediated non-canonical vs. Wnt3a-mediated canonical Wnt signals in determining the cell fate was controlled by the grafting density of HA on chitosan. Treatment with the inhibitor of canonical Wnt-associated signaling molecules suppressed the osteogenesis of MSC spheroids on chitosan. This study demonstrates that Wnt signaling of MSCs is distinct in 3D environment and is substrate-dependent. The convenient 3D platform may be used to examine the role of Wnt signaling in controlling MSC fate under different extracellular environments, and potentially applied to study stem cell behavior in regenerative medicine, normal development, and cancer.



## 1. Introduction

Many types of mammalian cells can form three-dimensional (3D) multicellular spheroids. Compared to the traditional two-dimensional (2D) monolayer culture, the cellular function and properties of 3D spheroids more faithfully replicate those of cells in vivo [1]. Multicellular spheroids from hepatocytes or other transformed cells are widely applied in cancer research and, more recently, in the emerging field of regenerative medicine [2–4].

Mesenchymal stem cells (MSCs) are multipotent cells that are able to differentiate into different lineages and expand while maintaining their undifferentiated state. Mammalian cell culture techniques previously developed to form multicellular spheroids were also applied to generate spheroids from MSCs. MSC spheroids generated from hanging drop or suspension culture had better antiinflammatory properties [5] and angiogenesis capacity [6]. Those formed on micropatterned substrates had higher efficiency of osteogenic and adipogenic differentiation [7]. Unlike the above systems often associated with the lack of an attachment surface, we have developed a unique system to generate dynamic 3D spheroids from MSCs [8]. In the system, MSCs were simply grown on biomaterial membranes made of chitosan, a natural, nontoxic, and inexpensive polymer obtained from deacetylation of chitin. The simplicity of the system allowed the real-time imaging of the spheroid forming process. On these membranes MSCs migrated very fast and were self-assembled into spheroids in 24 h. The spheroids were unique in a way that they remained adherent on the membranes, kept moving, and maintained the expression of stemness marker genes better than MSCs in 2D culture. Although MSC spheroids formed by different methods have been shown to possess higher differentiation capacities in general, the associated mechanisms remain unclear. In particular, these MSC spheroids do not seem to have the same properties and cell fate [5–11].

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http://dx.doi.org/10.1016/j.biomaterials.2013.03.031

Chitosan has amino group ($-NH_2$) that may chelate $Ca^{2+}$, and a process mimicking biomineralization could subsequently occur [12]. Hyaluronan (HA), a polyanion, is rich in animal tissues, e.g. articular cartilage, skin, and brain. The two molecules, i.e. chitosan and HA, are versatile and each as a scaffold has been reported to facilitate either chondrogenesis or osteogenesis [13,14]. In the current study, we used a series of membranes comprising chitosan and that grafted with different densities of HA (chitosan-HA) to generate dynamic MSC spheroids and to investigate the different cell fate of these substrate-derived 3D spheroids. We compared on these materials the effect of spheroid formation on the protein expression of two cell adhesion molecules, N-cadherin and OB-cadherin (cadherin 11) in basal, chondrogenic, or osteogenic induction media. The different chondrogenic vs. osteogenic differentiation potentials of MSC spheroids derived on different substrates were delineated and further linked to the activation of Smad2/3, ERK1/2, cytoplasmic and nuclear β-catenin, as well as several Wnt genes and proteins on these substrates. Specifically, we intended to explain the distinct properties of MSC spheroids by the substrate environment they were exposed to, which may predefine their differentiation capacities through Wnt signaling.

## 2. Materials and methods

### 2.1. Isolation and culture of adipose-derived MSCs

All procedures followed the ethical guidelines and were approved by the Animal Care and Use Committee of the University. MSCs were obtained from the adipose tissue of Sprague-Dawley rats (body weight from 350 g to 500 g). The adipose tissue was minced into several pieces and treated with 200 U/mL type I collagenase (Sigma–Aldrich) in Hank's buffered salt solution at 37 °C for 1 h with gentle agitation. The cellular pellet was washed by phosphate-buffered saline (PBS), centrifuged at 1500 rpm for 10 min and resuspended in PBS. The homogenate was cultured in basal medium, which consisted of Dulbecco's modified Eagle medium (DMEM)-low glucose/F12 (1:1) supplemented by 10% fetal bovine serum (FBS), 20 mM HEPES, 50 mg/mL bovine serum albumin fraction V, 10 mg/L L-glutamine, and 1% penicillin-streptomycin (antibiotics). Cell were grown on T75 flasks and maintained in a 37 °C/5% $CO_2$ incubator. The medium was refreshed twice every week. Cells of the third to the fifth passages were used in this study.

### 2.2. Analysis for the adipose-derived MSCs

The expression of surface markers was quantified by flow cytometry using anti-CD29, anti-CD31, anti-CD44, and anti-CD90 (BioLegend), anti-CD34, anti-CD45 (Santa Cruz Biotechnology), anti-CD73, and anti-CD105 (BD Biosciences) antibodies. Cells ($5 \times 10^5$) were washed twice with PBS, resuspended in 100 μl of PBS containing monoclonal antibodies and incubated for 30 min at 4 °C. The cells were then washed twice and resuspended in 500 μl of PBS. Fluorescence analysis was performed with a flow cytometer (FACS Caliber, BD Biosciences). The non-specific binding of the fluorescein isothiocyanate (FITC) and phosphatidyl ethanolamine (PE) conjugates were determined in control samples using a mouse IgG1-FITC and IgG1-PE negative control (Serotec). The stemness marker genes (Oct4, Sox2, and Nanog) were analyzed by real-time or quantitative RT-PCR (qRT-PCR). The multilineage differentiation capacities were conducted to verify the genuineness of the MSCs [15].

### 2.3. Preparation of chitosan membranes and those grafted with different densities of HA (chitosan-HA)

Chitosan powder was purchased from Sigma (USA). The molecular weight of the chitosan was 510 kDa. The degree of deacetylation measured by NMR was 77%. HA (sodium salt) was obtained from SciVision Biotech (Kaohsiung, Taiwan). The molecular weight was about 2500 kDa. Chitosan was dissolved in 1% acetic acid to obtain a 1% chitosan solution. The solution (300 μl) was coated on 1.5 cm-diameter coverslip glass placed in petri dish, where chitosan membranes formed after solvent evaporation in a laminar cabinet for 24 h. Sodium hydroxide (0.5 N) was added to the petri dish containing the membranes. The membranes were soaked for 30 min and washed three times with PBS. For the preparation of chitosan-HA membranes, 300 μl of HA solution containing different amounts of HA was added on each chitosan-coated coverslip glass so the initial amount of HA was 0.1, 0.5 or 2.5 mg per $cm^2$ of the membranes. The HA coated chitosan membranes on coverslip glass were then placed in each well of a 24-well tissue plate for further crosslinking. In each well, 150 μl of ethyl(dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) solution with a weight ratio of HA/EDC/NHS adjusted to 1:1.84:0.23 at pH 5.5 was added and shaken continuously for 48 h at 4 °C to crosslink HA on the surface [16]. After that, the membranes were washed fives times with PBS to remove the unbound HA and were later lyophilized. The amount of unbound HA in EDC/NHS solution after reaction and that in PBS washed solution were determined by the glucuronic acid assay described below. The chitosan membranes and those grafted with three different doses (from low to high) of HA were abbreviated as C, CH-L, CH-M, and CH-H respectively. The change in surface chemistry was verified by the attenuated total reflectance infrared (ATR-IR) spectroscopy. The surface water contact angle was measured by a static contact angle analyzer (FTA-1000 B, First Ten Angstrom Company, USA) at 25 °C and 70% relative humidity. The surface zeta potential was measured by a particle size and zeta potential analyzer (Delsa™ Nano, Beckman Coulter, USA) using laser light scattering and a flat solid cell. The surface topography and phase images were obtained with an atomic force microscope (AFM) (CP-II, Veeco, USA).

### 2.4. Glucuronic acid assay for the amount of surface bound HA and its release

The amount of HA conjugated on the chitosan surface was quantified by the glucuronic acid assay described in literature [17]. Chitosan-HA membranes were washed and the solution after wash was collected. An aliquot of 100 μl was hydrolyzed by 500 μl of 120 mM sodium tetraborate in concentrated sulfuric acid at 100 °C for 1 h. 40 μl of 2 mg/l m-hydroxydiphenyl reagent was then added to the reaction mixture. After 15 min of incubation, the absorbance of the resulting solution was measured at 520 nm. The amount of surface unbound HA (mg) was determined from the absorbance. The amount of grafted HA was calculated by deduction the unbound HA from the total HA.

The release of HA from membranes was measured in a period of 4 weeks in the basal medium by the glucuronic acid assay as described above. Chitosan-HA membranes were placed in each well of a 24-well tissue culture plate. 500 μl of the culture medium was removed from each well at different time points during the culture period, and followed by the addition of 500 μl fresh medium. The amount (mg) of HA released from a membrane was calculated and normalized to the initial amount (adsorbed). The results were shown as the cumulative release in percentage.

### 2.5. Cell seeding and spheroid formation

MSCs ($5 \times 10^4$ cells) were seeded on each membrane in 24-well tissue culture plates. Cells seeded in the culture well (tissue culture polystyrene, TCPS) served as the control. Cell morphology was examined by an inverted microscope (Leica, DMIRB). Cell grouping and spheroid formation were recorded at 3, 10, and 17 days. The dynamic process of spheroid formation was monitored by a real-time cell culture monitoring system (Astec, CCM-Multi, Japan). The tracks of spheroids and average migration rates during 0–3 days and 3–7 days were analyzed from the real-time images by the ImageJ software based on at least 30 spheroids in multiple video files. The expressions of OB-cadherin and N-cadherin were analyzed at 3, 10, and 17 days by Western blot. The gene expressions of Wnt3a, Wnt5a, and Wnt5b were analyzed at 3, 10, and 17 days by qRT-PCR. The protein expressions of Wnt3a, Wnt5a, and Wnt5b were analyzed by Western blot at 17 days. The expression of cytoplasmic and nuclear β-catenin was analyzed at 3 and 17 days by Western blot.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2013.03.031.

### 2.6. Induction of chondrogenic and osteogenic differentiation

To perform chondrogenic induction, the basal medium was replaced with the chondrogenic induction medium after MSCs were cultured on the membranes for 3 days. The induction medium was DMEM-high glucose containing 10% FBS, 10 ng/mL TGF-β3 (CytoLab/Peprotech, Rehovot, Israel), 0.1 μM dexamethasone (Sigma), 50 μg/mL ascorbate-2-phosphate (Sigma), 40 μg/mL L-proline (Sigma), 1% insulin–transferrin–selenium (ITS)-premix 100× (Sigma), and 1% antibiotics.

The induction medium was changed twice a week. After induction for 14 days (17 days from initial seeding), the expression of three chondrogenic markers genes [SRY-box containing gene 9 (Sox9), aggrecan (Aggr), and collagen type II (Coll II)] as well as the expression of collagen type I (Coll I) and collagen type X (Coll X) was analyzed by qRT-PCR. To assess the presence of cartilage-specific matrix components, cells at the end of culture period (17 days) were stained for glycosaminoglycans (GAGs) by Safranin-O (Sigma). Cells were also immunostained with the specific primary antibody for type II collagen (Chemicon). The immunofluorescence staining was detected using the FITC-conjugated goat anti-mouse IgG antibody (Chemicon) under a fluorescence microscope (Nikon Eclipse 80i, Japan). In the control group, MSCs on the membranes remained to be cultured in basal medium after the 3-day initial period (i.e. a total of 17 days in basal medium).

For osteogenic differentiation, the basal medium was replaced with the osteogenic induction medium after MSCs were cultured on the membranes for 3 days. The induction medium was α-MEM supplemented with 10% FBS, 10 μM β-glycerophosphate (Sigma), 50 μg/mL ascorbate-2-phosphate, 0.01 μM dexamethasone, and 1% antibiotics. The medium was refreshed twice a week. After induction for 14 days (17 days from initial seeding), the expression of runt-related transcription factor