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Effects of aspect ratios of stem cells on lineage commitments with and without induction media

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ABSTRACT

The present study is aimed to examine the shape effect on lineage commitment of stem cells in growth medium free of external chemical induction factors. Aspect ratios (ARs) of cells were controlled by micropatterns with cell-adhesive microislands of AR 1, 2 and 8 on the potent nonfouling background of poly(ethylene glycol) hydrogels, and the single stem cells were well shaped for 19 days. Mesenchymal stem cells (MSCs) derived from rat bone marrow were cultured in osteogenic medium, adipogenic medium, mixed coinduction medium, and also growth medium; alkaline phosphatase (ALP) and oil droplets were employed as indicators of osteoblasts and adipocytes, respectively. Those indicators were well observed in all of three induction media as early as day 7, and also in growth medium at a longer culture time till day 13. While a significant monotonic decrease of adipogenesis was observed with the increase of AR, a non-monotonic change of osteogenesis was found with optimal AR about 2. The relative gene expressions further verified the above findings. As a result, cell shape itself is an inherent cue to regulate stem cell differentiation, let alone with or without external chemical induction factors. Such a shape effect disappeared upon addition of a microfilament inhibitor cytochalasin D or a Rho-associated protein kinase (ROCK) inhibitor Y-27632. So, formation of cytoskeleton is necessary for the shape effect, and the ROCK-pathway-related cell tension is responsible for the shape effect on the lineage commitment of stem cells even in growth medium.

1. Introduction

Stem cells are, owing to their abilities of both self-renewing and multipotent differentiations, a very important type of seed cells in regenerative medicine [1–4]. It is thus meaningful to understand cues to regulate differentiation of stem cells. While various soluble factors [1,5–7] and chemical characteristics of substrates [8–12] have been found to influence cell differentiation, several physical cues have also been discovered such as stiffness [13–16], topography of substrate [17–22], and even cell size [23–26], shape [27–29], and cell–cell contact [26,30].

Aspect ratio (AR) is an important measurement of cell shape. Micropatterning techniques have been used to control the cell shapes. In induction media, the shape effects on differentiation of individual mesenchymal stem cells (MSCs) derived from bone marrow have been reported recently [27–29]. Both osteogenic and adipogenic differentiations of MSCs with different ARs were examined independently by Mrksich group [27] and Ding group [29]. Interestingly, while the two groups gave the same conclusion about a decreased adipogenesis with AR, different conclusions were drawn for osteogenesis: a monotonic increase of osteogenesis with AR was described by Mrksich group [27], and a non-monotonic change with optimal AR about 2 by Ding group [29]. While just three cases of AR 1, 1.5, and 4 were examined by Kilian et al. [27] when they made comparison between different ARs, 6 cases of AR 1, 1.5, 2, 4, 8, 16 were examined by Peng et al. [29] and those three cases of AR 1, 1.5 and 4 of Peng et al. [29] exhibited the same trend as the corresponding cases of Kilian et al. [27]. So, the data of these two articles are not contradictory with each other, although this story reminds us of importance of finding an appropriate variable range in the experimental design.

AR effects from the both groups were concluded in the presence of induction media. A fundamental question remains: whether or not the AR effect exists even without the induction chemicals, namely, just in growth medium. The answer is not trivial, but concerns whether or not the shape effect is an *inherent* cell geometry cue to regulate differentiation of stem cells. Kilian et al. [27] mentioned that “more than 95% of the MSCs did not show lineage specific staining in growth medium after 1 week”. We think that this might stand only for the short-time culture and hypothesize that a long time culture of stem cells with well

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controlled shapes might reveal the inherent shape effects even in growth medium.

It is, however, not easy to observe differentiation of single cells keeping a pre-designed shape for a long time. The micropatterning technique used by Kilian et al. [27] was put forwarded by Whitesides and his colleagues [31]. This technique is very useful and has been widely applied in many fields. For instance, the pioneering research to demonstrate the size-controlled cell adhesion by Chen et al. [32] and to reveal the cell size effect on differentiation of MSCs in induction medium by Chen group [23] were both enabled by the technique. However, this technique is hard to fix shapes of single cells for quite a long time, whereas long culture is usually required for cell differentiation. As honestly pointed out by Mrksich group in their impressive publication [27], "cells remained viable and constrained to the patterns for 1 week in culture, though at longer times the nonadhesive regions were degraded and cells escaped the pattern and proliferated". So the examination of cell differentiation for a longer time must be based upon an appropriate micropatterning technique with more potent nonfouling background. Our group has developed a technique to generate patterns of cell-adhesive peptide arginine-glycine-aspartate (RGD) on poly(ethylene glycol) (PEG) hydrogels. The bottleneck is how to make cell-adhesive RGD microdomains onto chemically inert nonfouling PEG hydrogels. This difficulty has eventually overcome by introducing a transfer lithography technique, and the methodology of the corresponding micropatterning technique has been published [33]. This material technique has been applied by us to reveal some of the fundamental issues in cell biology and biomaterials including cell adhesion [34,35], cell–cell interaction [26,30], and geometrical cues [26,29] on cell differentiation in induction media.

Herein we employed our unique material technique to examine the cell shape effect on lineage commitment of stem cells in growth medium. We planned to fabricate the cell adhesive RGD microislands with different ARs on nonfouling PEG hydrogels, as schematically presented in Fig. 1. Single stem cells might selectively adhere on the pre-designed microislands, and their shapes are required to remain persistently by the underneath microislands. In this paper, we will show the ability of our material technique to keep the cell shape as long as 19 days. MSCs on these micropatterned surfaces will then be exposed to osteogenic medium, adipogenic medium, mixed osteogenic and adipogenic coinduction medium, and also growth medium. We are curious about the answer whether or not the cell shape can regulate cell differentiation even under the growth medium free of soluble induction factors within this time scale.

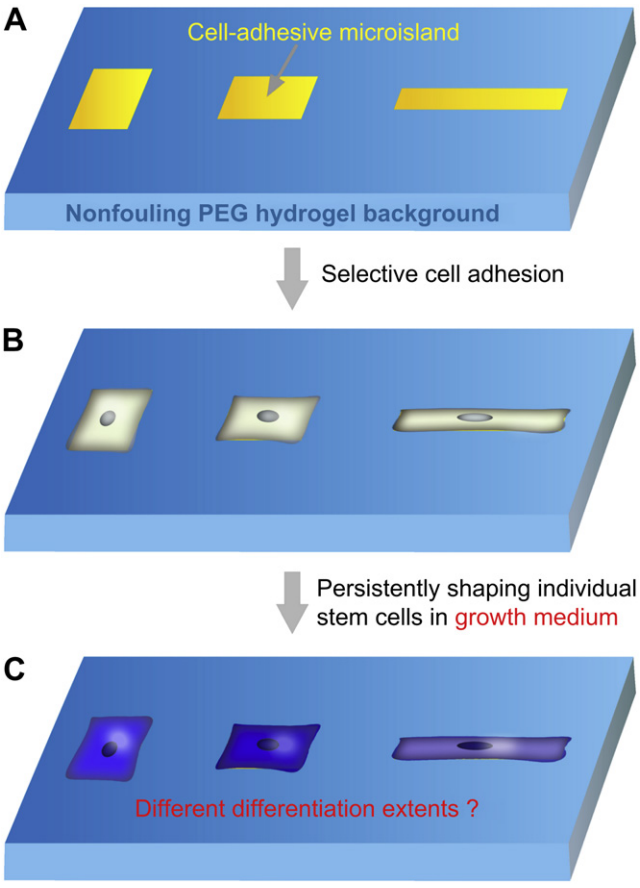

Fig. 1. Schematic presentation of the idea to explore the effects of aspect ratio on differentiation of stem cells in growth medium alone.

2. Materials and methods

2.1. Preparation of micropatterns with persistent cell adhesion contrast

We prepared gold micropatterns on glass using a traditional photolithography plus a lift-off technique, then transferred the gold micropatterns from glass to PEG hydrogels, following our previous protocol [33]. Yet, a different linker N, N′-bis(acryloyl) cystamine [36] (Sigma) was used in the present transfer lithography. In the transfer experiments, we first immersed the glass with Au microislands in the ethanol solution of 1 mM linker for 1 h, and the excess linkers were rinsed by ethanol 3 times, 15 min for each. The remaining linkers were bound to the Au microislands through the Au–S bond. Then, the mixture of the macromonomer poly(ethylene glycol) diacrylate (PEGDA) (MW 700, Sigma) and the initiator 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (D2959, Sigma) was cast on the glass surface. After UV exposure for 60 min, the PEG hydrogel was peeled off, obtaining the micropatterns of Au microislands on the nonfouling PEG hydrogels. The geometric cues such as ARs of adhesive microislands were determined by the masks used at the stage of photolithography.

2.2. Stem cell isolation and proliferation

MSCs were isolated from 7-day old neonatal Sprague Dawley (SD) rats. Briefly, the marrows of tibia and femur were flushed out and centrifuged at 800 rpm for 10 min, then resuspended in growth medium. The growth medium was prepared by addition of 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Gibco) and 10% fetal bovine serum (FBS, Gibco) into low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco). The isolated cells were cultured in a 5% CO_2 incubator at 37 °C. Non-adherent cells were removed and fresh growth media were added after 3 days. The cells were passaged upon almost confluence. Only early passage MSCs were used for later experiments.

2.3. Stem cell seeding on the micropatterns

Prior to cell seeding on micropatterns, we linked RGD molecules to the gold microislands. RGD is a typical peptide sequence to trigger specific cell adhesion and has been widely applied in biomaterial modifications [37–41]. In the present study, the PEG substrates with designed Au micropatterns were immersed in a 25 μM solution of cyclic peptide c(–RGDfK–)–OEG–$COCH_2CH_2SH$ (R: arginine, G: glycine, D: aspartic acid, f: D-phenylalanine, and K: lysine) (Peptides International, USA) in deionized (DI) water at 4 °C overnight, and excess linkers were rinsed by the DI water. The RGD molecules were covalently bound to gold microislands through the Au–S bond. After sterilization by 75% alcohol, the substrates were placed into 12-well plates (Corning). MSCs in the growth medium were seeded at a density of 5×10^4 cells per well. After 1 h, non-adherent cells were removed, and 1 ml fresh growth medium with aphidicolin (0.5 μg/ml, Sigma) were added in each well. After one day, the stem cells were cultured in fresh growth or induction media.

2.4. Stem cell culture and differentiation on the micropatterns

For the sole osteogenic induction (OM), the stem cells were cultured in the osteogenic induction medium containing 10% FBS, 50 μM ascorbic acid-2-phosphate, 10 mM β–glycerophosphate and 100 nM dexamethasone (Sigma) in high-glucose DMEM for 6 days (totally 7 days for cell culture plus the initial one day in the growth medium). In the case of sole adipogenic induction (AM), MSCs were exposed in the adipogenic induction medium (10% FBS, 1 μM dexamethasone, 200 μM indomethacin, 10 μg/ml insulin, and 0.5 mM methylisobutyxanthine (Sigma) in high-glucose DMEM) for 3 days and then in the adipogenic maintenance medium (10% FBS, 10 μg/ml insulin in high-glucose DMEM) for another 3 days.