Experimental Cell Research 337 (2015) 111–119

Contents lists available at ScienceDirect

Experimental Cell Research

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

Elastic hydrogel substrate supports robust expansion of murine myoblasts and enhances their engraftment

Ke Ding [a,*,1], Zhong Yang [b,1], Jian-zhong Xu [c,*], Wen-ying Liu [a], Qiang Zeng [a], Fang Hou [a], Sen Lin [d]

[a] Department of Pediatric Surgery, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu 610072, China
[b] Department of Clinical Hematology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
[c] Department of Orthopaedics, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
[d] Department of Anatomy and Histology & Embryology, Chengdu Medical College, Chengdu 610500, China

ARTICLE INFO

Article history:
Received 1 June 2015
Received in revised form
20 July 2015
Accepted 21 July 2015
Available online 22 July 2015

Keywords:
Cell culture
Myoblast
Cell transplantation
Cell therapy
Elasticity
Stemness

ABSTRACT

The application of satellite cell-derived myoblasts in regenerative medicine has been restricted by the rapid loss of stemness during *in vitro* cell expansion using traditional culture systems. However, studies published in the past decade have highlighted the influence of substrate elasticity on stem cell fate and revealed that culture on a soft hydrogel substrate can promote self-renewal and prolong the regenerative potential of muscle stem cells. Whether hydrogel substrates have similar effects after long-term robust expansion remains to be determined. Herein we prepared an elastic chitosan/beta-glycerophosphate/collagen hydrogel mimicking the soft microenvironment of muscle tissues for use as the substrate for satellite cell culture and investigated its influence on long-term cell expansion. After 20 passages in culture, satellite cell-derived myoblasts cultured on our hydrogel substrate exhibited significant improvements in proliferation capability, cell viability, colony forming frequency, and potential for myogenic differentiation compared to those cultured on a routine rigid culture surface. Immunochemical staining and western blot analysis both confirmed that myoblasts cultured on the hydrogel substrate expressed higher levels of several differentiation-related markers, including Pax7, Pax3, and SSEA-1, and a lower level of MyoD compared to myoblasts cultured on rigid culture plates (all $p < 0.05$). After transplantation into the tibialis anterior of nude mice, myoblasts that had been cultured on the hydrogel substrate demonstrated a significantly greater engraftment efficacy than those cultured on the traditional surface. Collectively, these results indicate that the elastic hydrogel substrate supported robust expansion of murine myoblasts and enhanced their engraftment *in vivo*.

1. Introduction

Satellite cells are recognized as true muscle stem cells (MuSCs) that are primarily responsible for muscle regeneration [1–5]. With advances in the fields of regenerative medicine and tissue engineering, satellite cells and their progeny, myoblasts, are attractive cell types for use in novel treatment strategies for multiple diseases such as progressive muscular dystrophy [6], myocardial infarction [7], and stress urinary incontinence [8].

For the success of applications employing myoblasts, the ability to obtain sufficient numbers of functional myoblasts is critical, and *in vitro* expansion of autologous or allogeneic satellite cells is the most practical way to acquire large number of myoblasts. However, once plated on standard tissue culture plastic, the stemness, a term used to describe stem cells' self-renewal and regenerative capacity, of myoblasts is lost rapidly, yielding cells with greatly diminished regenerative potential and therapeutic potency [9–11].

The stem cell niche, which consists of various soluble and insoluble factors, plays a crucial role in the regulation of stem cell fate [12–14]. The delivery of soluble factors to stem cells in culture has been the most extensively applied approach in efforts to stem cell fates [15,16]. However, in the past decade, more studies have investigated the effects of insoluble factors as well, and one such factor, substrate or matrix elasticity, was shown to have a remarkable influence on stem cell behavior [17–22]. Engler et al. [17] demonstrated that matrix elasticity directs stem cell lineage specification, and Gilbert et al. [23] indicated that even short-term cultivation on muscle-mimetic matrix can promote self-renewal and prolong the regenerative potential of MuSCs. Recently, Wang

* Corresponding authors.
E-mail addresses: dk1118@yeah.net (K. Ding), xjzspine@163.com (J.-z. Xu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.yexcr.2015.07.021
0014-4827/

et al. [24] reported the *in vitro* expansion of satellite cells on hydrogels with an elasticity similar to that of muscle tissue can maintain cells in a quiescent state and inhibit their differentiation. However, to our knowledge, the behavior of satellite cells after long-term cultivation on an elastic substrate remains unknown.

Polymer gel systems that perfectly mimic the softness of various tissues have been developed for investigating the effects of substrate rigidity on a variety of cell types in culture [23–25]. As a substrate capable of mimicking the elasticity of muscle tissue, chitosan/beta-glycerophosphate/collagen (C/GP/Co) hydrogel is a polymer gel system that has demonstrated both good histocompatibility and an elastic modulus similar to that of the murine *anterior tibialis* (TA) [26–28]. The elasticity of C/GP/Co hydrogel can be adjusted simply by changing the proportion of collagen in the C/GP/Co mixture before polymerization, and thus, these hydrogels can be engineered to precisely simulate the elastic microenvironment of muscle tissues.

To investigate the influence of an elastic hydrogel substrate on the long-term *in vitro* expansion of satellite cell-derived myoblasts, we prepared a hydrogel system with an elastic modulus similar to that of the murine TA and applied it as a substrate for myoblast culture. We evaluated the proliferation of myoblasts over 20 passages as well as the differentiation capacity and engraftment efficacy of myoblasts after 20 passages on our hydrogel substrate.

2. Materials and methods

2.1. Fabrication of hydrogel substrate

The protocol for C/GP/Co hydrogel fabrication was described in detail in our previous study [28]. To adjust the material elasticity, we prepared a series of mixtures in which the volume ratio of collagen was varied: 5:1:0 (no collagen), 5:1:3, 5:1:6, and 5:1:12. After gelation, the elastic moduli of the gels ($n=5$ per composition) were quantified using an Instron 3342 (Instron) as previously reported [25]. According to the results of this experiment, the optimal C/GP/Co volume ratio that achieved the elasticity that most precisely matched that of murine muscle tissue was applied in the preparation of hydrogel substrates for myoblast culture.

2.2. Myoblast isolation and culture

Satellite cell-derived myoblasts were isolated from limb muscles of enhanced green fluorescent protein (eGFP) transgenic mice (C57BL-Kα-β/actin-eGFP) using a pre-plating protocol described previously [28]. The protocol and use of these mice for cell isolation was approved by the committee on the care and use of animals of Sichuan Provincial People′s Hospital. Cells were then cultured and expanded in growth medium (GM) consisting of Ham′s F_{10} (Hyclone) medium, 20% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (P/S, Sigma-Aldrich), and 10 ng/mL basic fibroblast growth factor (bFGF, Hyclone).

The experimental group was defined as myoblasts cultured on the hydrogel substrate, and the control group as myoblasts cultured on standard tissue culture polystyrene plates. The numerical subscript in the expression P_X indicates the passage number of myoblasts. Unless otherwise noted, myoblasts were analyzed at passage 20 (P_{20}) in this study.

2.3. Myoblast proliferation

The proliferation of P_2, P_{10}, and P_{20} myoblasts in the experimental and control groups was evaluated using the cell counting kit-8 assay (CCK-8, Dojindo) as described previously [28]. GM without myoblasts was used as a negative control, and proliferation was measured in six replicate wells per group and passage number.

2.4. Trypan blue exclusion assay

The percentage of dead cells in culture was assessed using the Trypan blue exclusion assay following previously published procedures [29]. Briefly, cells were harvested, pelleted, and resuspended. After incubation with 0.4% Trypan blue solution (Sigma-Aldrich) for 5 min, 10 μL of Trypan blue/cell mixture was applied to a hemocytometer for cell counting. Stained (dead or dying) and non-stained (live) cells were counted separately under a microscope (IX70, Olympus). We counted at least 300 cells in three replicates for each group and then calculated the percentage of stained cells.

2.5. Colony-forming assay

EGFP-labeled myoblasts were sorted by flow cytometry (FACSCalibur, BD Biosciences) and then seeded in the inner 60 wells of 96-well plates at 1 cell per well. GM was added to the inner 60 wells (100 μl/well), and to decrease the evaporation rate, 100 μl of phosphate-buffered saline (PBS) was added into each of the outer 36 wells. Only wells in which exactly one cell was seeded were included for subsequent observation. The plates were then transferred to an incubator and cultured routinely. The growth of myoblasts was observed daily, and the colony forming frequency (CFF) was calculated after 5 days in culture. The CFF was calculated as the percentage of wells in which colonies formed out of the total number of wells in which only one cell was seeded, and the volume of medium remained adequate throughout the cultural period without considerable evaporation. Three parallel replicates were analyzed for each group.

2.6. Myogenic differentiation assay

When the cell reached 70% confluency, the GM was changed to myogenic induction medium containing Dulbecco′s Modified Eagle Medium (DMEM, Hyclone), 2% horse serum (Hyclone), and 1% P/S (Sigma-Aldrich). After a 5-day myogenic induction period, the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma-Aldrich). To evaluate the cells′ capability of myogenic differentiation, we assessed the fusion index and percentage of myotubes with five or more nuclei [30,31]. The fusion index was calculated as the ratio of nuclei present in multinucleated myotubes divided by the total number of DAPI-labeled nuclei.

2.7. Immunocytochemical staining

Myoblasts on sterile glass coverslip were washed with PBS three times, fixed with 4% formaldehyde (Sigma-Aldrich) at room temperature for 20 min, washed another three times with PBS, permeabilized for 15 min by treatment with 0.2% Triton X-100 (Sigma-Aldrich) in PBS, and then blocked with 3% bovine serum albumin (BSA, Hyclone) in PBS containing 0.1% Triton X-100 for 1 h. Primary antibodie solutions were added to the cells for incubation overnight at 4 °C. The next day the cells were washed three times with PBS and incubated with secondary antibody solution for 1 h in darkness at room temperature. After three additional rinses in PBS, the nuclei were counterstained with DAPI (1:1000) for 1 min before mounting. Images were visualized and captured using a microscope (IX70, Olympus) and then analyzed with Image pro-plus 6.0 software. The primary antibodies included those to paired box protein 7 (Pax7, 1:400, Santa Cruz Biotechnology), paired box protein 3 (Pax3, 1:400, Santa Cruz