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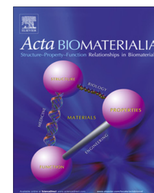

Three-dimensional hydrogel scaffolds facilitate in vitro self-renewal of human skin-derived precursors

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ABSTRACT

Skin-derived precursors (SKPs) are multipotent cells with dermal stem cell properties. These easily available cells possess the capacity to reconstitute the skin in vivo, as well as a broader differentiation potential in vitro, which endows them with great prospects in regenerative medicine. However, the present authors' group and others previously found that adult human SKPs (hSKPs) expanded deficiently in vitro, which largely counteracted their research and practical values. Taking the physiological micro-environment of hSKPs into consideration, the authors sought to establish a hydrogel scaffold-based three-dimensional (3-D) culture system for hSKPs in the present study. After comparing their morphology, growth characteristics, signature gene expression and differentiation potential in different hydrogels, the present authors found that a chemically defined hyaluronic acid and denatured collagen-based hydrogel system that mimicked the natural niche of hSKPs in the dermis could alleviate hSKP senescence, support hSKP proliferation as spheres, while largely retaining their properties and potential. This study suggested that recapitulating the in vivo stem cell niche by providing them with 3-D extracellular matrix environments could help them achieve better self-renewal in vitro. In addition, the animal-origin-free and biocompatible 3-D hydrogel system will certainly benefit fundamental research and clinical applications of hSKPs in the near future.

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1. Introduction

Multipotent human adult stem cells have received extensive attention, owing to their potential value in the field of regenerative medicine. Skin-derived precursors (SKP), first identified in the rodent dermis [1] with dermal stem cell properties [2], are considered a promising somatic stem/progenitor population, because they can not only reconstitute the skin [2], but also differentiate between several other lineages, such as neurons, bone cells and muscle cells [2–4]. Mouse and rat SKPs have already shown their capacity to repair damaged nervous system, bones and skeletal muscles [2,4,5]. Human SKPs (hSKPs) with similar properties have also been isolated from multiple human body areas [6], and they could have been an ideal stem cell source for regenerative medicine, since they are very easy to obtain. Unfortunately, adult hSKPs showed drawbacks in long-term self-renewal in vitro, as indicated by some researchers [3,7]. Separated from their natural microenvironment, human adult SKPs go senescent quickly under routine spheroid culture and show a drastic decrease in their sphere-forming ability, which is known to be one of the common properties of many somatic stem cell types, such as neural stem cells and some cancer stem cells [7,8]. Therefore, to realize their potential therapeutic value, in vitro expansion methods for hSKPs must be optimized.

The physiological microenvironment of a stem cell (the niche) controls the stem cell behavior to a great extent, because it provides the cell with not only the physical structure, but also mechanical integrity and biochemical activity [9]. A major element of a three-dimensional (3-D) stem cell microenvironment is the hydrated, crosslinked networks of extracellular matrix (ECM), which is usually absent in conventional two-dimensional cell culture systems. To provide the stem cells with more biomimetic culture conditions, various biomaterials have been used to create

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3-D scaffolds. Hydrogels, whether natural or synthetic, are water-swollen polymer networks resembling soft tissue in many respects [9], and they are the most investigated biomaterials for 3-D stem cell expansion or differentiation. Embryonic stem cells, for example, showed greatly enhanced propagation and self-renewal on 3-D nanofibrillar surfaces [10,11], and embryoid body formation could be manipulated within hydrogel scaffolds, with the desired sizes and differentiation biases [12]. Expansion and directed differentiation of human mesenchymal stem cells in hydrogel scaffolds with different composition, mechanical properties, topography and degradability have been studied extensively in recent years [13–17]. In addition, increasing types of adult stem cells, such as neural stem cells (NSCs) [18], muscle stem cells [19] and adipose-tissue-derived stem cells [20–22], are being explored to grow or differentiate in 3-D hydrogel matrix. Notably, biocompatible hydrogel scaffolds loaded with functional cells or growth factors succeeded in the recovery of various tissues in animal models [23,24], showing encouraging prospects for human tissue engineering.

The present study sought to establish a hydrogel-based 3-D expansion system to support the in vitro self-renewal of human foreskin SKPs. Several candidate hydrogel materials were selected. First, methylcellulose (MC) is widely used in clonal cell culture, although it is not a natural ECM component. MC semi-solid culture was reported to effectively inhibit hSKP sphere over-aggregation, which is detrimental for these cells [7]. Second, alginate scaffolds are used to encapsulate and support the spherical expansion of NSCs [25,26]. Given the similarity of SKPs to NSCs in the spheroid morphology, Nestin expression and differentiation potential [1,6], it is possible that alginate hydrogels might also be suitable for hSKPs. AlgiMatrix (AM), a commercial alginate-based hydrogel scaffold, was used. Third, SKPs are derived from the dermis, which is replete with collagen and elastin fibers, filled with gel-like matrix mainly composed of glycosaminoglycans (most notably hyaluronic acid), proteoglycans and glycoproteins [27]. Therefore, collagens and hyaluronic-acid-based hydrogels were also of interest. As a representative material of such hydrogels, the commercially available HyStem (HS) culture system was used. The above materials were compared with several considerations. First, spheroid growth of hSKPs is supposed to be maintained, since it is one of the most important characteristics of hSKPs. Second, proliferation of hSKPs within hydrogels is another critical evaluating factor. Lastly, the multipotency of hSKPs should not be impaired.

2. Materials and methods

2.1. Skin sample processing and initial cell culture

Human foreskin samples were derived from voluntary circumcisions with informed consent, and the protocol was approved by the Ethical Committee of the Institute of Zoology, Chinese Academy of Sciences. The foreskin sample processing procedure was described previously [8]. Briefly, freshly excised foreskin samples were washed with sterile saline. After the removal of subcutaneous fat and vessels, skin samples were subjected to additional intensive washing. Subsequently, stripped skin pieces were digested with 5 mg ml^{-1} Dispase (Gibco, NY, USA, www.lifetechnologies.com) in phosphate-buffered saline (PBS) overnight at 4 °C. After the removal of the epidermis, dermal pieces were further smashed and incubated in 5 mg ml^{-1} collagenase type IV (Gibco) at 37 °C for ~5 h. Single cell suspension was harvested by pipetting, filtering and washing. Freshly isolated dermal cells were plated in petri dishes (BD Falcon, CA, USA, www.bd.com) and cultured at 37 °C with 5% CO_2, with a plating density of 10^6 cells ml^{-1}. Cell expansion medium was DMEM/F12 (Gibco) supplemented with 20 ng ml^{-1} epidermal growth factor, 40 ng ml^{-1} basic fibroblast growth factor (bFGF) (both from Peprotech, NJ, USA, www.peprotech.com) and 2% B27 (Gibco).

2.2. Subculture of hSKPs in routine and 3-D conditions

The culture medium was changed on day 3 of initial culture, when cell aggregates were seen. On day 6, hSKP spheres were formed, while other cell types were gradually eliminated through death or attachment to the culture dish. On day 6, hSKP spheres were trypsinized with 0.25% trypsin (Invitrogen) and pipetted into single cells. Cells were re-plated into routine culture medium as control groups or different matrices. For control groups, 2×10^6 cells were plated into each well of Costar Ultra-low attachment 6-well plates (Costar, USA, http://www.corning.com/lifesciences) with 4 ml culture medium. For MC groups, DMEM/F12 medium with 2% MC (Sigma, www.sigmaaldrich.com) was used instead of normal DMEM/F12 medium, while cell plating density and the volume of culture medium were the same as those in control groups. AlgiMatrix™ 3-D culture 6-well plates were purchased from Lifetechnologies (NY, USA, www.lifetechnologies.com) and were used following the manufacturer's instructions. In each AM well, 2×10^6 cells were plated, with a total volume of 4 ml culture medium. HyStem™-C Cell Culture Scaffold Kits were purchased from Sigma and also used following the manufacturer's instructions. Briefly, 2×10^6 cells were mixed well with 2.5 ml premixed solution of kit components and pipetted into a well of the Costar Ultra-low attachment 6-well plate before gelling. An additional 2.5 ml culture medium with doubled growth factors was given to each HS well after the cells were firmly embedded in the HS hydrogels. During the following days, culture medium in all groups was changed every 3 days. For medium change, hSKPs in control and MC groups needed to be centrifuged while the medium was directly changed in AM and HS groups.

2.3. Cell harvest for following assessments

To harvest cells for the following assessments, hSKPs in control and MC groups were directly centrifuged and collected. To retrieve cells from each AM hydrogel, 5 ml dissolving buffer containing 55 mM sodium citrate and 0.15 M NaCl (pH 6.05) were used to digest the gel for 20 min at room temperature. Each HS hydrogel was incubated with 3 ml DMEM/F12 containing 300 U ml^{-1} collagenase and 100 U ml^{-1} hyaluronidase at 37 °C for 2 h before hSKP spheres were collected. hSKPs recovered from each group were used directly for immunofluorescent staining or trypsinized into single cells for staining, RNA extraction or differentiation.

2.4. Differentiation of human foreskin-derived SKPs

After 15 days of culture, hSKP spheres were trypsinized and pipetted into single cells. Cells were seeded into 11 mm coverslips pre-coated with poly-D-lysine/Laminin (Sigma) in 24-well plates. The differentiation medium was DMEM/F12 with 5% fetal bovine serum (FBS) for the first 3 days, and then cells were subjected to three different media for neuronal, glial (Schwann cell) and spontaneous differentiation. Neuronal differentiation medium was Neurobasal medium (Gibco) with 50 ng ml^{-1} human nerve growth factor, 50 ng ml^{-1} human brain-derived neurotrophic factor (BDNF), 10 ng ml^{-1} Neurotrophin-3 (NT-3) (all from Sino Biological, China, sinobiological.cn) and 1% FBS (Hyclone, Australia, www.hyclone.com). The glial differentiation medium was neurobasal medium with 10 ng ml^{-1} heregulin (Peprotech), 4 μM forskolin (Sigma), 1% N2 supplement (Gibco) and 1% FBS. The spontaneous differentiation medium was DMEM/F12 with 1% FBS. The medium was half-changed every 3 days, and cells were

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