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Nasal ectomesenchymal stem cells: Multi-lineage differentiation and transformation effects on fibrin gels

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ABSTRACT

Ectomesenchymal stem cells (EMSCs) are novel adult stem cells derived from the cranial neural crest. However, their stemness and multi-lineage differentiation potential on three-dimensional fibrin gels has not yet been explored. The objective of this study was to investigate induced differentiation of EMSCs on fibrin gels and their remodeling effects on the scaffolds during the induced differentiation process. The results indicated that $CD133^+$/$nestin^+$/$CD44^+$ EMSCs were extensively distributed in the lamina propria of the nasal mucosa. The passaged cells could be induced to differentiate to a greater degree into neurons, Schwann cells and osteoblasts on three-dimensional fibrin gels than on two-dimensional glass slides. More importantly, the induced Schwann cells and osteoblasts exerted channelized and calcified remodeling effects, respectively, on the fibrin gels. Thus, these reshaped scaffolds have desirable biological properties, such as good cell adhesion, biocompatibility and guidance over the cell behavior, providing a tissue-committed niche for specific tissue generation.

1. Introduction

Tissue damage and organ failure due to traumatic injuries or degenerative diseases pose a great challenge in the field of tissue engineering therapy. Following attempts to restore or improve the biological functions of damaged tissues, numerous biomaterials have been developed to construct three-dimensional (3D) scaffolds that allow functional tissues to be generated by adjusting and guiding cell proliferation and differentiation.

To date, the biomaterials most widely used for tissue engineering can be roughly classified into two categories according to their origins, i.e., natural materials (collagen, hyaluronic acid, chitosan, fibrin, etc.) [1–3] and synthetic materials (polyglycolic acid, polylactic acid, poly DL-lactic co-glycolic acid, and so on) [4,5]. Among these materials, fibrin is notable due to its good cell adhesion, high level of cell proliferation, ability to support a uniform distribution of cells and excellent biocompatibility [6,7]. Most importantly, unlike synthetic materials and other natural materials, such as xenogenic collagen, which may trigger inflammatory responses, fibrin can be produced from the patient's own blood by a reaction between fibrinogen and various coagulation factors, thus lowering the possibility of an inflammatory reaction [8].

Traditional tissue engineering has focused on 3D scaffolds that contain cells and bioactive factors. These 3D scaffolds provide a spatial environment that mimics the *in vivo* conditions, and the added bioactive factors often serve as the constructors of a favorable microenvironment for the seeded cells. Thus, the functionalized 3D scaffolds provide a beneficial platform that guides all types of cell behaviors (migration, proliferation and differentiation, etc.) in the desired direction [9–11]. However, this investigational approach has stagnated at the laboratory stage due to various challenges, such as intrinsic flaws in the materials, the difficulty in delivering factors, poor cell phenotypic stability, long treatment duration and high cost [12,13]. Therefore, these limitations have encouraged the development of an alternative approach that relies on extracellular matrix (ECM). ECM, which is produced by the resident cells, is a complex of proteins and polysaccharides that exists in a state of dynamic equilibrium with its resident cells. ECM is constantly being built, reshaped and degraded in response to

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changing environmental conditions [14]. ECM can not only serve as the supporting material for cells but also interact with the resident cells to regulate cellular functions, such as migration, proliferation and differentiation.

Over the past few decades, ECM derived from decellularized tissues (tissue-derived ECM) has been extensively explored for tissue engineering and regenerative medicine due to its versatile advantages described above. However, it suffers from intrinsic flaws, such as immunogenicity and pathogen transfer from allogenic and xenogenic tissues, as well as the scarcity of autologous tissue [15]. In contrast, cell culture-derived ECM is a more appealing option due to its multiple advantages. For instance, cell culture-derived ECM is superior to tissue-derived ECM in several aspects. It avoids the undesired immune response that may be induced by allogenic and xenogenic tissues, it provides a solution to the scarcity of autologous tissues, and it prevents pathogen transfer [16,17]. However, cell culture-derived ECM alone suffers from its own problems, such as a lack of mechanical strength and difficulties during handling. Thus, scaffolds fabricated by the deposition of ECM secreted by the resident cells in 3D scaffolds derived from natural/synthetic biomaterials, which are known as cell culture-derived ECM scaffolds, may offer a promising alternative due to the combined merits of both components. Specifically, the tunable mechanical strength of the scaffold material provides the suitable rigidity and spatial environment necessary for various tissues, whereas the ECM provides biochemical and biophysical cues to guide cellular functions, which is often absent in a 3D scaffold alone. In short, cell culture-derived ECM scaffolds hold great potential in tissue engineering and regenerative medicine.

In this study, we aimed to investigate the differentiation potential of ectomesenchymal stem cells (EMSCs) in fibrin gels and the remodeling effects of EMSCs on 3D scaffolds of fibrin gel, especially the calcified- and channelized-reformation of the fibrin gels resulting from EMSC seeding under induced differentiation conditions (osteogenic and Schwann cell differentiation, respectively).

EMSCs are a multipotent progenitor population derived from the cranial neural crest, which could be isolated from the oral mucosa [18], dental tissues [19] and the olfactory and respiratory mucosa niche [20]. As a type of adult stem cell, EMSCs possess several unique advantages. For example, they can be non-invasively harvested from adult patients [21], while avoiding ethical issues raised by the use of embryonic stem cells (ESCs). Because EMSCs originate from the neural crest, they have the potential to differentiate into various cell phenotypes, such as osseous cells, neurons and glial cells [22–24]. Due to these favorable properties, EMSCs were employed in the present work as the seeded cells in fibrin gels.

The inspiration to investigate bone regeneration is due to its constant, high demand in the clinical setting. Calcified remodeling of the fibrin gel may provide an additional option for osteogenic differentiation and bone repair. Similarly, suitable scaffolds are vital for nerve regeneration following peripheral nerve injury. Due to the challenges associated with the current clinical treatments, identifying effective systems to direct neuron axonal growth is an urgent demand. It has been well documented that an intimate relationship exists between neurons and glia, both during development and in response to injury [25]. Schwann cells (SCs), the major glial cells of the peripheral nervous system, can facilitate organization of the neural ECM, form axon myelination, and provide structural and trophic support for axonal regrowth [12,26]. Hence, an investigation of the reshaping effect on the fibrin gels caused by SCs differentiated from EMSCs is of great significance for the development of an appropriate scaffold for nerve tissue regeneration.

Although there have been previous reports on osteogenic differentiation and bone repair [27], articular cartilage generation [15], and nerve tissue engineering [12] via cell culture-derived ECM along with various biomaterials, few studies have addressed the differentiation potential of EMSCs in fibrin gels, and no previous report has investigated the calcified- and channelized-remodeling effects on these scaffolds by the differentiated cells. Therefore, we intend to address these aspects to establish a promising platform for bone regeneration and nerve tissue engineering.

2. Materials and methods

2.1. Reagents

DMEM medium, F12 medium, neurobasal medium, B27, and fetal bovine serum (FBS) were purchased from Invitrogen (Paisley, UK). Antibodies, including goat polyclonal anti-CD133 (prominin1) was purchased from Santa Cruz (California, USA); Rabbit polyclonal anti-Sall-4, anti-Sox-1, anti-Sox-10, anti-collagen-I, anti-Runx2, anti-osteocalcin, anti-osteopontin, anti-integrinβ1 and mouse monoclonal anti-CD44, anti-CD133, anti-vimentin, anti-NF-200, anti-GAP43, anti-synapsin, anti-synaptotagmin, anti-laminin, and anti-fibronectin were purchased from Abcam (Cambridge, UK). Rabbit polyclonal anti-nestin and mouse monoclonal anti-S100β, anti-p75, anti-GFAP, anti-CNPase, anti-β-actin, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cy3(FITC)-conjugated secondary antibodies, all-trans retinoic acid (ATRA), dexamethasone, β-glycerophosphate disodium, L-ascorbic acid 2-phosphate, forskolin (FSK), rat fibrinogen and alizarin red S were purchased from Sigma–Aldrich (St. Louis, MO, USA). Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor-AA (PDGF-AA), β-heregulin (HRG), sonic hedgehog (SHH), and brain derived neurotrophic factor (BDNF) were purchased from PeproTech (PeproTech, USA).

2.2. Immunofluorescence staining of rat nasal mucosa

All of the animal procedures in this study were approved by the Animal Care and Ethics Committee of Jiangsu University, and the Guidelines for Animal Research were strictly followed. A total of 5 Sprague–Dawley rats weighing 120–140 g were provided by the animal center of Jiangsu University. The rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (0.05 g/kg) and then perfused with heparinized saline (10 IU/ml) followed by 4% paraformaldehyde (PFA, ProSciTech). The nasal olfactory and respiratory mucosa were dissected together with the nasal septum and turbinates and then post-fixed in 4% PFA overnight. The samples were decalcified for 7 days in 15% EDTA/4% PFA for cryosectioning. The decalcified tissues were washed with PBS and then dehydrated with gradient sucrose solutions. After embedding the tissues in OCT compounds, they were cryosectioned into coronal serial sections (25 μm in thickness) using a Leica cryomicrotome. These sections were washed in PBS for 10 min and permeabilized with 0.1% TritonX/1% bovine serum albumin (BSA) in PBS. Primary antibodies, including anti-CD133, anti-nestin, and anti-CD44, diluted in 1% BSA/PBS were applied to the sections for incubation overnight at 4 °C. After the incubation, the sections were washed three times in PBS and then incubated with the Cy3-conjugated secondary antibody diluted in 1% BSA/PBS for 1 h at room temperature. The tissue sections were washed three times in PBS and then counterstained with Hoechst 33342 to visualize all nuclei in the tissues. The parallel negative controls were subjected to the same procedures without primary antibodies. The stained tissues were observed under an immunofluorescence microscope (Axio Observer, ZEISS, Germany).

2.3. Cultured EMSCs derived from the nasal mucosa

The lower one third of the nasal septum and inferior nasal concha were dissected from anesthetized Sprague–Dawley rats weighing 120–140 g. The mucosa were stripped and minced into pieces (0.5–1 mm^3). The mucosa pieces were explanted onto the bottoms of plastic flasks and then incubated for 2 h with a few fresh FBS. After the explants attached to the bottoms of the plastic flasks, fresh medium (1:1 DMEM/F12, 10% FBS) was added. The explants were cultured in an atmosphere of 5% CO_2 at 37 °C. The medium was replaced every 3 days, and the adherent cells, namely EMSCs, were passaged once a week.

2.4. Detection of stem cell markers

For detecting the expression of stem cell markers, the primary and passaged EMSCs cultured on poly-L-lysine (PLL)-coated glass cover slips were fixed using 4% paraformaldehyde. The cells were immunofluorescence-stained with antibodies against various stem cell markers, including Sox1, Sall4, Snail, Sox10, S100β, CD133, nestin, vimentin, CD44 and integrin β1. Parallel negative controls were subjected to the same procedures without primary antibodies. The stained EMSCs were observed under an immunofluorescence microscope (Axio Observer, ZEISS).