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The isolation and *in situ* identification of MSCs residing in loose connective tissues using a niche-preserving organ culture system

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ABSTRACT

Mesenchymal stem cells (MSCs) have been discovered in a multitude of organs, but their distribution and identity are still uncertain. Furthermore, loose connective tissue (LCT) is dispersed throughout virtually all organs, but its biological role in tissue homeostasis is unclear. Here, we describe a unique organ culture system to explore the omnipresence and *in situ* identity of MSCs among the LCTs. This culture system included the use of the fibrin hydrogel coupled with dynamic culture conditions, using native LCTs obtained from various organs as starting materials. This culture allowed MSC outgrowth into the hydrogel to be robustly supported, while maintaining the structural integrity of LCTs during *in vitro* culture. Subcultured outgrown cells fulfilled the minimal requirements for defining MSCs on the basis of clonogenicity, multipotency, and immunophenotypic characteristics. *In vitro* label-retaining assay demonstrated that the numbers of mobilized and proliferated cells *in situ* increased in the pericapillary region and expressed both MSCs and pericytes markers, indicating that the *in situ* identity of MSCs represents a certain population of pericapillary pericytes. Our results indicate that this culture system affords a unique strategy for both isolating MSCs and recapitulating their niche in LCTs.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent progenitor cells with the capacity to differentiate into mesodermal cells and to retain extensively proliferative ability during *in vitro* culture [1,2]. MSCs were first isolated from bone marrow (BM) and were referred to as colony-forming units of fibroblast (CFU-F) or marrow stromal cells [2]. Now, these cells have been discovered in a multitude of organs including skeletal muscle (SM), adipose tissue (AT), trabecular bone, synovia, dermis, dental pulp, lung, and blood vessel (BV) [3–13]. Although studies on MSCs have been extensive, the distribution and *in situ* identity of MSCs within their native tissues remain unsolved [14].

Recent evidence indicates that a rare population of pericytes may in fact be the native MSCs [13–15]. Pericytes are named for their perivascular location and they contribute to vascular stabilization and tissue regeneration [16]. Following appropriate stimulation, these cells are liberated from the vascular wall, and they migrate to an injury site, proliferate, and replace the damaged tissues. Thus, pericytes have been suspected to be, or at least to include, progenitor cells that can differentiate into other cell types [8,13–15,17]. Recently, cultured pericytes isolated from a multitude of organs were shown to share biological properties with MSCs [12,13]. Despite the growing circumstantial evidence that certain populations of pericytes might be the MSCs, no convincing

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evidence yet exists that pericytes have key features in common with *in situ* MSCs.

Pericytes and MSCs are apparently slow-cycling *in vivo* and their *in situ* frequencies are intrinsically very low, which makes these cells difficult to detect in the quiescent state of the tissue [14]. Furthermore, only limited markers are available to identify MSCs or pericytes this point. However, all previous attempts to define MSCs or pericytes used these limited markers and quiescent tissue [4,9,10,13]. Recently, we [7] and others [18,19] have developed hydrogel-supported 3-dimensional (3D) organ culture systems that can recapitulate the stem cell niche *in vitro*. These culture systems can maintain the structural and functional integrity of the stem cells niche, thereby allowing the adoption of *in vitro* 3D organ culture system for defining *in situ* identity and niches of stem cells residing in various organs.

Loose connective tissue (LCT) is a distinctive type of connective tissue characterized by loosely arranged extracellular matrix (ECM) fibrils and abundant microvessels [20]. Its function is primarily to protect and connect various parts of organs. The LCT covers the outermost surface of solid organs, BV, and peripheral nerve (PN) as an adventitia or epineurium. Moreover, LCT provides an internal framework within solid organs, dividing them into small anatomical units, and delivers vascular and neural networks into small anatomical units. Recent studies have demonstrated that the vascular wall retains MSCs whose potentials extend far beyond vascular cell lineages [10,11]. Interestingly, these cells resided around the vasa vasorum in adventitia, which is a typical example of LCT [11]. The present report highlights our hypothesis that MSCs may not be restricted to the adventitia of BV, but may be widely distributed among LCTs in a multitude of organs.

In this study, we adopted a fibrin hydrogel-supported 3D organ culture system to determine whether MSCs are widely distributed among LCTs from SM, AT, BV, PN, and intestinal submucosa (IS). Our intention was develop a unique strategy for isolating MSCs from LCTs on the basis of 3D organ culture. Like a provisional matrix *in vivo*, the fibrin hydrogel used for 3D organ culture could be used as a biomimetic substratum for selective outgrowth and proliferation of MSCs residing in LCTs. After recovery of outgrown cells from the hydrogel, we determined whether these cells fulfilled the criteria established for MSCs. Finally, using our culture system, we enabled to define the localization and phenotypic characteristics of *in situ* mobilized and proliferated MSCs residing in LCTs.

2. Materials and methods

2.1. Hydrogel-supported 3D organ culture of LCT

All human LCT samples ($n = 32$) were obtained from cadaveric donors (15–56 years, mean 49 years) after approval and by following the guidelines of the ethical committee at Inje University School of Medicine. Large amounts of tissues were obtained from a multitude of organs including peri- and inter-fascicular LCTs of SM ($n = 9$), peri- and inter-lobular LCTs of AT ($n = 10$), adventitial LCTs of BV ($n = 5$; 3 abdominal aorta, 2 inferior vena cava), epineurial LCTs of PN ($n = 5$), and submucosal LCTs of IS ($n = 3$). The LCT fragments were selectively separated from visible blood vessels, nerves, and blood clots using a dissecting microscope (Fig. 1A). The LCT fragments were washed with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA), minced into 2–3 mm^3 fragments, and suspended in a thrombin (Sigma, St. Louis, MO) solution dissolved in Dulbecco's modified eagle medium (DMEM; Invitrogen) at a concentration of 1 NIH U/ml. This LCT fragment suspension was mixed with the same volume of fibrinogen (Greencross, Suwon, Korea) dissolved in DMEM at a concentration of 5 mg/ml, and this mixture was transferred into a culture dish. After incubation in a humidified chamber at 37 °C for 2 h to polymerization, growth culture media were added to culture dish to cover the hydrogel-encapsulated LCTs. Growth culture medium was composed of 90% DMEM:Ham's F12 (1:1) mixture (Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), 10 ng/ml epidermal growth factor (PeproTech, Seoul, Korea), 2 ng/ml basic fibroblast growth factor (PeproTech), 10 ng/ml insulin-like growth factor

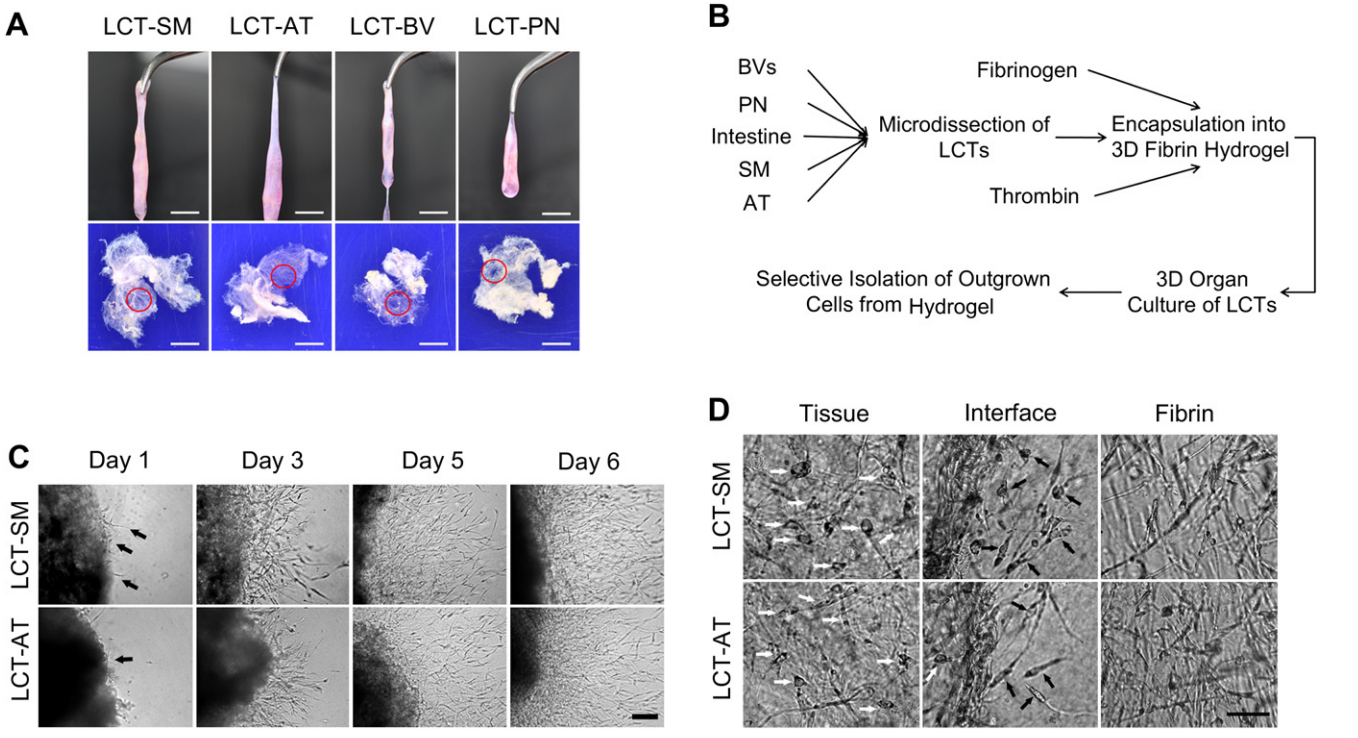

Fig. 1. Three-dimensional (3D) organ culture of loose connective tissues (LCTs). A: Macroscopic photographs of LCTs harvested from various organs. A LCT (circle) was selectively microdissected from surrounding tissue. B: Schematic diagram of fibrin hydrogel-supported 3D organ culture system of LCTs. C: Phase-contrast photographs of outgrown cells (arrow) derived from LCTs embedded in hydrogel. Outgrown cells were spindle-shaped and occupied a large area of hydrogel. D: Phase-contrast microscope visualized increased cell density within the embedded LCTs and cells migrated into interfaces and the fibrin hydrogel. Cell numbers were increased inside the LCTs and showed a similar morphology to that of the outgrown cells. SM, skeletal muscle; AT, adipose tissue; BV, blood vessel; PN, peripheral nerve. Scale bar = 1 mm (A), 200 μm (C), and 100 μm (D).