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Single cell sorting identifies progenitor cell population from full thickness bovine articular cartilage

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SUMMARY

Objective: To date, no approved clinical intervention successfully prevents the progressive degradation of injured articular cartilage that leads to osteoarthritis (OA). Stem/progenitor cell populations within tissues of diarthrodial joint have shown their therapeutic potential in treating OA. However, this potential has not been fully realized due in part to the heterogeneity of these subpopulations. Characterization of clonal populations derived from a single cell may help identify more homogenous stem/progenitor populations within articular cartilage. Moreover, chondrogenic potential of clonal populations from different zones could be further examined to elucidate their differential roles in maintaining articular cartilage homeostasis.

Method: We combined Fluorescence-activated cell sorting (FACS) and clonogenicity screening to identify stem/progenitor cells cloned from single cells. High-efficiency colony-forming cells (HCCs) were isolated, and evaluated for stem/progenitor cell characteristics. HCCs were also isolated from different zones of articular cartilage. Their function was compared by lineage-specific gene expression, and differentiation potential.

Results: A difference in colony-forming efficiency was observed in terms of colony sizes. HCCs were highly clonogenic and multipotent, and overexpressed stem/progenitor cell markers. Also, proliferation and migration associated genes were over-expressed in HCCs. HCCs showed zonal differences with deep HCCs more chondrogenic and osteogenic than superficial HCCs.

Conclusion: Our approach is a simple yet practical way to identify homogeneous stem/progenitor cell populations with clonal origin. The discovery of progenitor cells demonstrates the intrinsic self-repairing potential of articular cartilage. Differences in differentiation potential may represent the distinct roles of superficial and deep zone stem/progenitor cells in the maintenance of articular cartilage homeostasis.

Introduction

Cartilage lesions are a fairly common problem in orthopedic practice. However, as an avascular and aneural tissue, articular cartilage has minimal intrinsic healing ability[1]. More often, most macroscopic cartilage lesions not only cause local tissue damage, but initiate whole joint progressive cartilage degeneration, which will ultimately leads to osteoarthritis (OA)[2,3]. Stem cell-based treatments have been explored for enhancing cartilage repair in degenerating joint for the past few years[4–6]. Evidence has emerged on the existence of MSCs-like cells from the synovium, articular cartilage, infrapatellar fat pat[7–9], and other tissues within articular joints. These cells can be primed towards chondrogenic differentiation both *in vitro* and *in vivo*, thus might represent possible candidates to maintain normal turnover of cartilage as well as to restore damaged cartilage upon joint lesions. Nevertheless, more complete understanding of their reparative behaviors is needed to further explore their therapeutic potential.

Adult mesenchymal stem cells (MSCs), or cartilage chondroprogenitors, are known to reside residing in hyaline tissue and have been shown to be highly clonogenic, multipotent, and chemotactic[10–12]. These tissue stem/progenitor cells are able to migrate towards local injury sites, where they proliferate and differentiated as needed to replace damaged tissue[13,14]. Unlike MSCs, which are

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able to differentiate into multiple tissue types in different organ systems, tissue progenitor/stem cells are typically only capable of generating limited tissue types for local tissue regeneration, especially the tissue of their origin. Stem/progenitor cells in articular cartilage are an example of the latter cell type, one that is able to undergo multi-lineage differentiation, but *in situ* and in normal physiological conditions is lineage restricted to differentiate into hyaline cartilage-producing chondrocytes.

Chondrogenic progenitor cells (CPCs) were first discovered by Dowthwaite *et al.*, who identified them to be a subpopulation of superficial zone cells for appositional growth of articular cartilage[15], which have enhanced affinity to fibronectin and highly expressed stem cell-associated factor Notch-1. Koelling *et al.* have also found CPCs in articular cartilage during later stages of human OA[16], these cells were highly migratory towards damaged cartilage tissue and repopulated in repair tissue. Grogan *et al.* later examined the distribution of stem cells markers (Notch-1, Stro-1, VCAM-1), and found inconsistency between stem-cell marker expression and stem cells distribution, thus concluded that these stem cell markers may not be useful to identify progenitors in cartilage. Some other studies also showed stem/progenitor cells overexpressed stem cell surface markers (CD105, CD166)[17] and were capable of Hoechst 33,342 dye exclusion as a side population, characteristic of stem cells[18]. Moreover, we previously found migrating CPCs strikingly proliferating on the articular surface post traumatic injuries in an *in vitro* bovine osteochondral explant impact model in response to multiple alarmins released by necrotic cells[19]. Another study also showed that injured bovine cartilage induces migration of Notch-1 positive cells to the surface of damaged region[20].

Despite the evidence that these cells might represent a putative cartilage progenitor cell maintaining the homeostasis of the articular joint, only a few studies thus far have identified a homogeneous single cell-derived clonal sub-population within the normal articular cartilage[21]. Full characterization of stem/progenitor cell potential requires the generation of genetically identical populations from a single progenitor[22]. Otherwise, the phenotypic "stemness" may actually result from a heterogeneous pool of cells with different origins. Williams *et al.* has demonstrated clonal cartilage progenitor cells have distinct phenotype from full-depth chondrocytes, as well as different telomerase activity[23]. In addition, where progenitors from articular cartilage normally reside within extracellular matrix is still not clear and worthy further investigation.

In the present study, we describe, for the first time, a single cell clonogenicity screening technique to identify progenitor cells in healthy articular cartilage. This technique allows isolation of progenitors from the superficial 1/3 as well as deep 2/3 of full thickness cartilage, with distinct differences in differentiation potency. Genetic and functional characteristics of the high-efficiency colony-forming cells (HCCs) reveal their similarities with adult stem/progenitor cells.

Materials and methods

Cartilage tissue harvesting and cell isolation

Fresh stifle joints from young adult cattle (15–24 months old) were obtained from a local abattoir (Bud's Custom Meats). Articular cartilage was harvested from the femur condyle using a 6 mm biopsy punch [Fig. 1(B)] and rinsed in Hank's Balanced Salt Solution (Invitrogen, California, USA) supplemented with 100 U/μl penicillin, 100 μg/ml streptomycin, and 2.5 μg/μl fungizone. Full thickness cartilage biopsy samples were minced into fine pieces and digested overnight with 0.25 mg/ml collagenase type 1 and pronase E (1:1) (Sigma–Aldrich, St. Louis, MO) dissolved in culture medium in a shaking incubator overnight (0.25 mg/ml each). When needed, a customized apparatus was used to separate the superficial 1/3

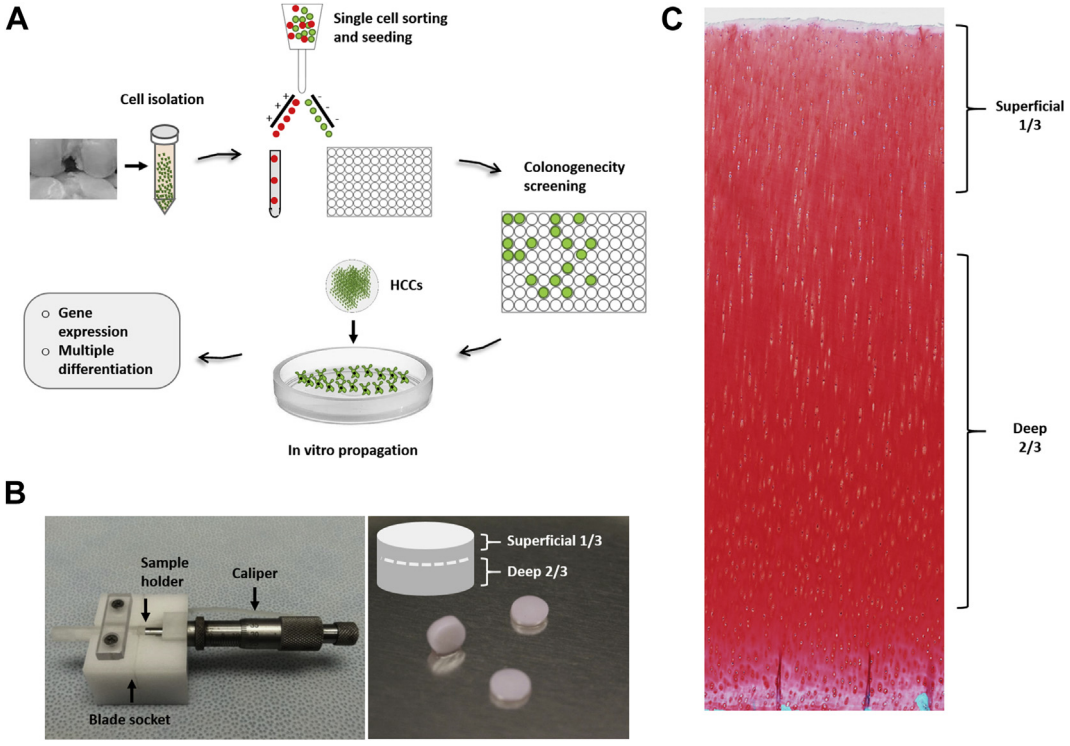

Fig. 1. Experimental schematics and customized apparatus. A) Flow diagram representing the methodology for isolating and characterizing cartilage progenitor cells. B) A customize-made apparatus for separating superficial and deep cartilage from 6 mm cartilage biopsy, with a caliper, and sample holder, and a blade socket. C) Histological image showing full thickness bovine articular cartilage (Scale bar represents 500 μm).