ORIGINAL RESEARCH PAPER

In vitro formation of lacuna structure by human dermal fibroblasts co-cultured with porcine chondrocytes on a 3D biodegradable scaffold

Xia Liu · Guangdong Zhou · Wei Liu · Wenjie Zhang · Lei Cui · Yilin Cao

Abstract Dermal fibroblasts (DF) possess chondrogenic differentiation potential but whether DF, or a subpopulation of DF, can form a typical cartilage structure in culture is unknown. In this study, human DF were co-cultured with porcine articular chondrocytes on a biodegradable scaffold of polylactic acid/polyglycolic acid. Histological analysis demonstrated that some DFs can be induced to form cartilage lacuna structure showing the existence of a chondrogenic subpopulation of human DF. Moreover, the 3D-co-culture system can serve as an optimal model for directing stem cell differentiation in vitro.

Keywords Chondrocytes · Chondrogenesis · Co-culture · Dermal fibroblasts · Scaffold of polylactic acid on polyglycolic acid

Introduction

The chondrogenic phenotype, characterized by cartilage-related molecule expression at both gene and protein levels, is one of the recognized criteria for examining the multipotency of mesenchymal stem cells (MSC) isolated from bone marrow, adipose tissue, skeletal muscle, etc. Particularly, cartilage structure formation, for example lacuna structure formation, is a more convincing test of the true nature of isolated "stem cells". However, in vitro induction of chondrogenic differentiation of cells is more difficult than the induction of other lineage differentiation. Suboptimal differentiation conditions often result in controversial conclusions, especially in a heterogeneous population that contains few stem cells. For example, some previous work has demonstrated that dermal fibroblasts (DF) possessed chondrogenic differentiation potential by upregulating their gene and protein expression of both collagen II and proteoglycan when they were cultured in an aggrecan coated dish or on a demineralized bone matrix scaffold (French et al. 2004; Mizuno and Glowacki 2005); whereas other studies failed to exhibit the chondrogenic phenotype of DFs even they were cultured in chondrogenic induction medium (Jones et al. 2002, 2004). Nevertheless, no studies have ever demonstrated that DF, or a subpopulation of DF, can truly form cartilage structure under optimal conditions of chondrogenic induction.

Previously, we have established a reliable in vivo chondrogenic differentiation model by co-transplanting bone marrow stromal cells (BMSC) with articular chondrocytes to prove the chondrogenic potential of BMSC, particularly lacuna formation ability (Zhou et al. 2005). In the current study, we modified the

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system by co-culturing human DF with porcine articular chondrocytes (PAC) on a 3D biodegradable scaffold in vitro to determine the chondrogenic potential of human DF.

Materials and methods

Cell culture

All experimental protocols involving human subjects were approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine. Fresh human foreskin specimens were obtained from five donors who underwent a routine circumcision procedure, and human DF were similarly isolated and cultured as previously described (Harford 2002). Porcine articular chondrocytes (PAC) were isolated from five hybrid pigs, 8 weeks old, and cultured as previously described (Zhou et al. 2005).

Preparation of cell-scaffold constructs and grouping design

A cylinder-shaped scaffold, 9 mm diam and 2 mm ht, was prepared using polylactic acid (PLA)-coated polyglycolic acid (PGA, 12 mg) unwoven fibers as previously described (Zhou et al. 2006). Human DF at passage 2 and PACs at passage 1 were mixed at a 1:1 ratio, total 10^7 cells, were then seeded onto each PLA/PGA scaffold and cultured in regular medium composed of DMEM plus 10% (v/v) FBS ($n = 5$). Equivalent amounts of PACs and DF were, respectively, seeded and cultured in regular medium as controls ($n = 5$ for each group). In addition, DF-scaffold constructs cultured in chondrogenic induction medium (DMEM plus 10% (v/v) FBS, 40 ng dexamethasone/ml, 10 ng TGF-β1/ml and 50 ng IGF-1/ml) were used as a factor-induced control ($n = 5$). After 5 days culture, a portion of the specimens were examined by scanning electron microscope (SEM) as described previously (Kim et al. 2004). All groups were kept in culture for 8 weeks.

Histology and immunohistochemistry

The specimens in all groups were fixed, embedded in paraffin, and cut into 5 μm sections. The sections were stained with hematoxylin and eosin and Safranin-O to evaluate histological structure and cartilage matrix deposition in the engineered tissue. Expression of type II collagen was detected by a mouse anti-human type II collagen monoclonal antibody (Santa Cruz) and a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (DAKO) followed by color development with diaminobenzidine tetrahydrochloride (DAB).

Immuno staining of human nucleolus

In order to examine the distribution of DF in co-cultured specimens, the sections were incubated with a mouse anti-human-nucleolus antibody (Chemicon) (Nunes et al. 2003). After washing with PBS, the sections were further incubated with a Rhodamine-labeled anti-mouse antibody or with a HRP-conjugated anti-mouse antibody (DAKO) followed by color development with DAB. In addition, Hoechst staining was applied to reveal all cell nucleoli. The percentage of DF in co-culture specimens was calculated based on the Rhodamine stained cell number versus the Hoechst stained cell number.

Results

Cells, scaffolds, and cell-scaffold constructs

DF revealed spindle-shaped morphology in culture (Fig. 1A), whereas PAC were round- or polygon-shaped with a smaller size compared to DF (Fig. 1B). Both DF and PAC proliferated and attached well to PGA fibers with abundant extracellular matrix production (Fig 1E, F), indicating good biocompatibility between the cells and the scaffolds.

Gross view, histology, and collagen II expression

After 8 weeks of culture in vitro, the constructs made from pure chondrocytes maintained their original size upon gross examination (Fig. 2A) and formed homogenous cartilage with lacuna structure formation and collagen II expression (Fig. 2E, I). The constructs seeded with a mixed cell population also formed cartilage-like tissue with relatively small size when examined grossly (Fig. 2B). Histology confirmed cartilage formation with collagen II expression

Fig. 1 Cells, scaffolds, and cell-scaffold constructs. (**A**) Human dermal fibroblasts at passage 2; (**B**) primary porcine articular chondrocytes (PACs); (**C**) gross view of polylactic acid/polyglycolic acid scaffold; (**D**) SEM examination of the scaffold; (**E**) microscope examination of the mixed-cell-scaffold construct; (**F**) SEM examination of the mixed-cell-scaffold construct. The scale bars = 100 μm
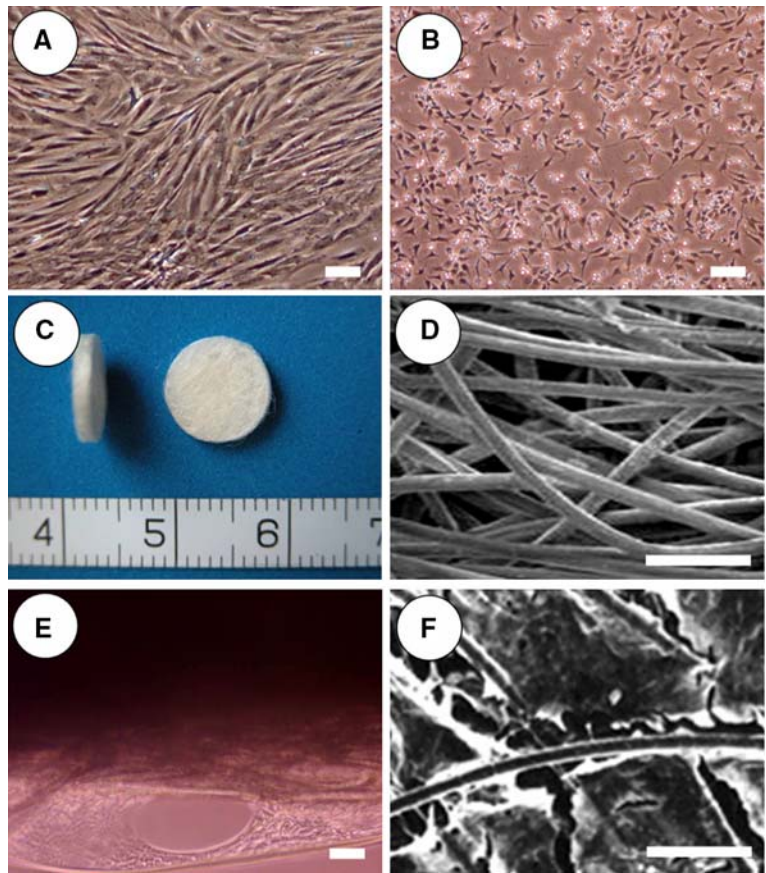
in the peripheral area (Fig. 2F, red rectangular area; 2J) but not in the central area (Fig 2F, blue rectangular area), indicating that most of the DF did not form cartilage tissue. In the DF control group of chondrogenic induction, the specimens shrunk markedly and failed to form cartilage-like tissue from both gross (Fig. 2C) and histological (Fig. 2G) examinations and they expressed a small amount of collagen II (Fig. 2K). The specimens in non-induced DF control group formed the smallest size tissues (Fig 2 D) without cartilage-like tissue structure (Fig. 2H, L).

Distribution and chondrogenic differentiation of DF in co-cultured specimens

To identify DF from the cartilage-like tissue formed by the mixed cell population, anti-human-nucleolus antibody was used, which clearly demonstrated the presence of approximately 50% human cells (Fig. 3 A) compared to whole nucleoli staining by Hoechst which stained both cell types (Fig. 3B, C). Furthermore, using this specific identification method, a few

percent of DF-derived cells had formed a lacuna structure in the peripheral area (Fig. 2F, red rectangular area; 2J) of the cartilage-like tissue formed by the mixed cell population (black arrows in Fig. 3D). Noticeably, many of the DF did not form lacuna structures in the same area as shown by the blue arrows in Fig. 3D, indicating that only a small amount of DF-derived cells could be converted to chondrocytes. Further supporting evidence was provided by the Safranin-O staining that demonstrated cartilage matrix production in the lacuna structures formed by DF (Fig. 3F, black arrows). As expected, in the central area (Fig 2F, blue rectangular area), neither lacuna formation (Fig. 3E) nor cartilage matrix production (Fig. 3G) was observed although a large quantity of DF were located in this area.

Discussion

To our best knowledge, this is the first study reporting the existence of a small number of cells in human dermis which are capable of forming lacuna structure
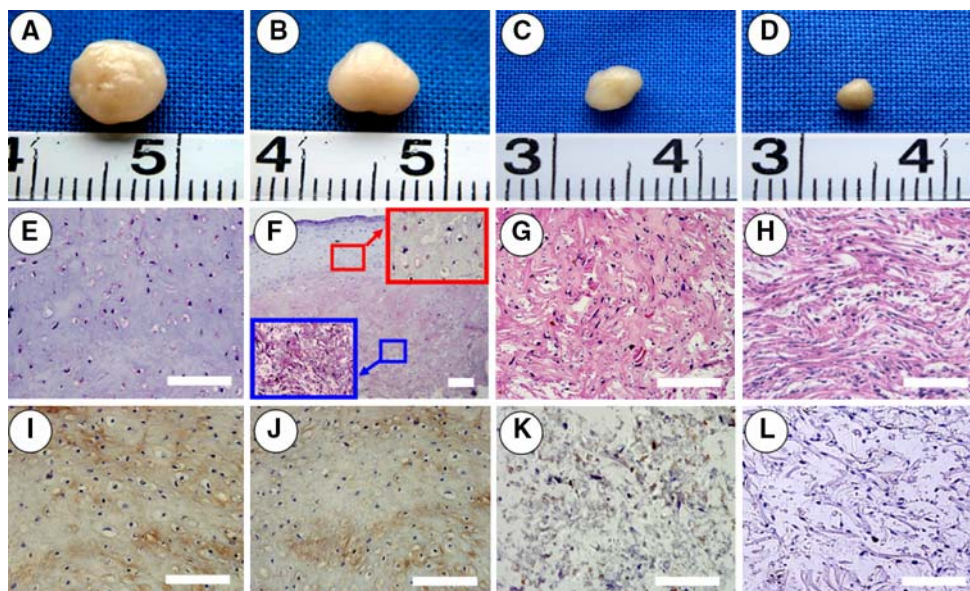
Fig. 2 Gross view and histology of in vitro cultured specimens at 8 weeks. (**E–H**) staining with hematoxylin and eosin; (**I–L**) immunohistochemical staining of collagen II. The gross view of specimens in porcine articular chondrocyte group (**A**), in mixed cell group (**B**), in chondrogenically-induced human dermal fibroblast group (**C**), and in non-induced human DF group (**D**). Histology shows cartilage-like tissue formation in PAC group (**E, I**) and in peripheral area of mixed cell group (**F**, *red rectangular region*, or **J**). No cartilage formation was observed in the central area of the mixed cell group (**F**, *blue rectangular region*), in chondrogenically induced HDF group (**G, K**) or in non-induced HDF group (**H, L**). The scale bars = 200 μm

(a characteristic structure of cartilage) when cultured in appropriate conditions. The convincing evidence of chondrogenic potential from this work further supports the true existence of multipotent stem cells in human DF as reported by others (Toma et al. 2001; Bartsch et al. 2005). In addition, our recent work also confirmed that few clonal cells from human dermis are multipotent and contain chondrogenic differentiation potential (Chen et al. 2007).

The controversy regarding the chondrogenic differentiation potential of human DF is possibly due to the suboptimal differentiation environment utilized in previous studies. As reviewed by Heng et al. (2004) many factors, including protein-based cytokines and growth factors, extracellular matrix, as well as cell–cell interactions, are important for directing the differentiation of stem cells towards a chondrogenic lineage in culture. The current 3D co-culture system with chondrocytes as a co-cultured cell type could well mimic the chondrogenic niche by providing inducible factors, unique cartilage matrices, and cell–cell interactions for chondrogenic differentiation, which is different from the methods reported by others (French et al. 2004; Mizuno and Glowacki 2005; Jones et al. 2002, 2004).

The heterogeneity of human DF could be another possible reason contributing to the foregoing controversy. High-density culture of chondrogenic cells is required for in vitro chondrogenesis (DeLise et al. 2000; Tsonis and Goetinck 1990). Our recent work showed that the clone forming ratio of human DF was below 2%, and only about 6% (3/48) of the clonal cells exhibited chondrogenic potential (Chen et al. 2007), indicating that the frequency of chondrogenic cells in the pooled HDF population is extremely low. Thus, there is likely an insufficient chondrogenic cell density for in vitro chondrogenesis (especially for cartilage lacuna formation), even using pellet culture as a model. The necessary cell density, however, can be easily achieved through the co-culture of human DF with mature chondrocytes.

In summary, this study has demonstrated the existence of a subpopulation of human DF that can be induced to form specific cartilaginous structures. In addition, our established 3D-co-culture system may
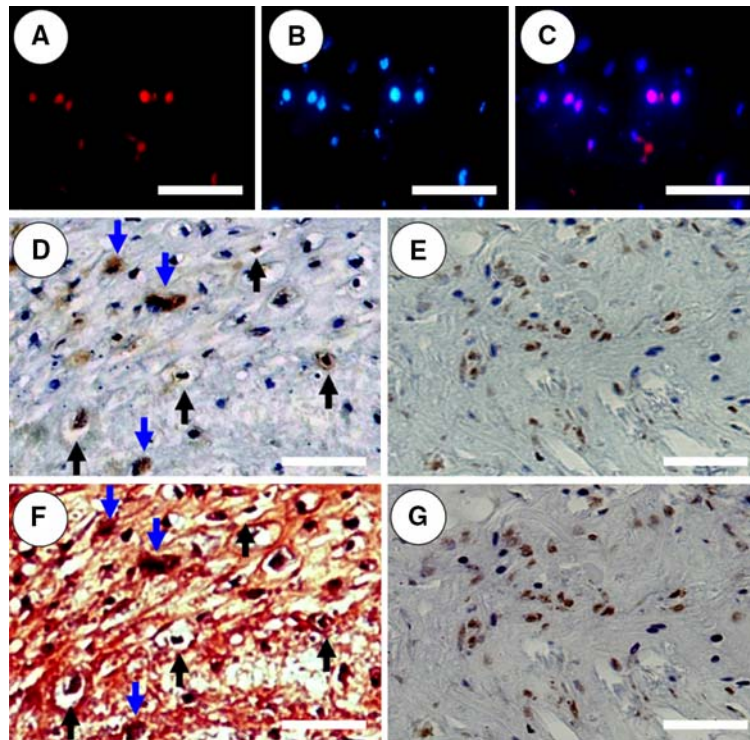
Fig. 3 Distribution of human dermal fibroblasts (HDFs) and their formed lacunas in 3D-co-culture specimens. Anti-human-nucleolus antibody identifies nucleoli of human DF (A, *red*), and Hoechst staining reveals all nucleoli (B, *blue*). Merged picture of A and B shows the distribution of human DF (*purple*) in total stained nucleoli (C). In the peripheral area of the cartilage-like tissue formed by mixed cell population (D and F), some human DF form lacuna structures (D, *black arrows*) and produce cartilage matrix (F, *black arrows*); nevertheless, other DF neither form lacuna nor produce the matrix (*blue arrows*). In the central area of the mixed cell formed tissue (E and G), neither lacuna nor cartilage matrix production is observed, although many DF (*brown colored*) are found in this area. (D and E): Immnuohistochemistry for human nucleolus; (F and G): Safranin-O staining for D and E, respectively. The scale bars = 100 µm

serve as an optimal model for directing stem cell differentiation in vitro.

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