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Isolation and Chondroinduction of a Dermis-Isolated, Aggrecan-Sensitive Subpopulation With High Chondrogenic Potential

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Objective. **To develop a process that yields tissue-engineered articular cartilage constructs from skin-derived cells.**

Methods. **Dermis-isolated, aggrecan-sensitive (DIAS) cells were isolated using a modified rapid adherence process. The chondrogenic potential was measured by quantitative reverse transcriptase–polymerase chain reaction, enzyme-linked immunosorbent assay, and immunohistochemistry. Filamentous actin (F-actin) and vinculin organization was detected using fluorescence microscopy.**

Results. **The rapid adherence process led to a selection of DIAS cells, <10% of the entire population. DIAS cells displayed greater chondroinduction potential, as evidenced by the formation of large numbers of chondrocytic nodules on aggrecan-coated surfaces. In addition, these cells showed higher gene expression and protein production in terms of chondrocytic markers when compared with unpurified dermis cells. Similar patterns of F-actin and vinculin organization were observed between DIAS cells and chondrocytes. Three-dimensional constructs from chondroinduced DIAS cells produced greater amounts of cartilage matrix than constructs from the rest of the dermis populations.**

Conclusion. **These findings show a series of steps that work together to form tissue-engineered articular cartilage constructs using DIAS cells. Since skin presents a minimally invasive, relatively abundant cell source for tissue engineering, this study offers evidence of an efficient and stable technique to form cartilage constructs for future cartilage regeneration with autologous cells from skin.**

Articular cartilage is an avascular, aneural, and alymphatic tissue supported by subchondral bone. This tissue undergoes frequent damage, which does not normally self-repair, resulting in permanent defects or mechanically inferior fibrocartilage (1). Attempts to develop clinically useful procedures to repair damaged articular cartilage include arthroscopic lavage and debridement (2), bone marrow stimulation techniques (3), and osteochondral transplantation (4). While some of these approaches show promise, most cartilage repair techniques lead to fibrocartilage formation, long-term donor morbidity in multiple donor sites, and/or cartilage degeneration after a temporary relief of symptoms (2–4). Thus, treatment options are limited, and long-term outcomes are uncertain at present. Articular cartilage tissue engineering is a promising therapeutic approach that combines cells, biomaterials, and environmental factors to promote functional restoration.

Challenges related to the cellular component of tissue engineering include cell sourcing, as well as expansion and differentiation. Recent studies have demonstrated that autologous chondrocyte implantation significantly diminishes pain and improves function (5). This technology motivates a demand for analogous cell-based strategies for cartilage repair, such as those attempted via the combination of biodegradable or biocompatible scaffolds with chondrocytes (6,7). Based on these studies, however, it is unlikely that a sufficient supply of chondrocytes will be available for clinical applications. Various adult tissues, including bone marrow (8,9), muscle (10,11), and adipose tissue (12,13), have been investigated as alternative cell sources for cartilage tissue engineering, with varying degrees of success.

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Skin is the largest organ in the body and is easily accessed with minimal harm to the donor. Dermis is therefore considered to be one of the best autologous source organs from which to isolate stem/progenitor cells for future therapeutic applications, not only in the replacement of skin (14), but also as a potential alternative cell source for other organs. Recent evidence indicates that dermis-derived cells can generate multiple lineages, including neurons, smooth muscle cells, and adipocytes (15). Dermis-derived cells may prove to be a useful alternative cell source for articular cartilage tissue engineering, since they have formed colonies of a chondroblastic phenotype (16–18). However, other studies demonstrated the existence of heterogeneous dermal fibroblast subpopulations with distinct characteristics (19,20). This strongly suggests that a limitation exists when using heterogeneous cells from the dermis for articular cartilage tissue engineering. It is therefore relevant to ask which subset of cells from the heterogeneous population of dermis-derived cells is the genesis of the chondrocytic phenotype.

Extracellular matrix (ECM) coatings on plastic surfaces have been used to provide proper surface conditions for chondroinduction. Types I and II collagen, hyaluronan, and fibronectin were found to play functional roles in regulating chondrogenic differentiation of mesenchymal progenitor cells (21–24). Our group has previously shown that various fibroblast cell lines are capable of chondrogenic differentiation when cultured on aggrecan-coated surfaces (ACS) (25). We therefore propose the hypothesis that a subpopulation of skin cells from the dermis can be isolated, and that this subpopulation is responsible for the phenomenon of ACS-assisted chondroinduction. We have termed this subpopulation dermis-isolated, aggrecan-sensitive (DIAS) cells.

Up to now, most articular cartilage regeneration strategies have been scaffold-based. However, there are disadvantages that come with using either natural or synthetic scaffold materials (26). Many synthetic polymers can induce inflammatory responses or create a local environment unfavorable to the biologic activity of cells. On the other hand, the major problem associated with natural polymer scaffolds is reproducibility. With the understanding that challenges exist in finding scaffolds with the correct balance between biofunctional and physical properties, we recently developed a novel self-assembly approach to fabricate 3-dimensional (3-D) articular cartilage constructs in vitro (27). This strategy avoids the disadvantages of scaffold materials. In addition to isolating DIAS cells from mixed dermis populations, we therefore propose the hypothesis that chondroinduced dermis cells can self-assemble into 3-D cartilage-like constructs in vitro.

MATERIALS AND METHODS

DIAS cell isolation and chondroinduction in monolayer culture. Full-thickness abdomen skin specimens were obtained from 5 goats, separated from underlying adipose tissue, and digested with 0.5% Dispase at 4°C overnight. The epidermis was then removed by scraping with a blade, and meticulously cleaned to remove all adipose tissue and blood coagulates in vessels (28). The dermis specimens were then washed, minced, and digested in phosphate buffered saline (PBS) containing 200 units/ml type II collagenase (Worthington, Lakewood, NJ) at 37°C for 15 hours with gentle rocking. After incubation, the cell suspensions were diluted at a ratio of 1:4 with expansion medium (Dulbecco's modified Eagle's medium [DMEM; Gibco, Grand Island, NY] supplemented with 10% fetal bovine serum [FBS; BioWhittaker, Walkersville, MD], 1% penicillin–streptomycin–amphotericin B [BioWhittaker], and 1% nonessential amino acids [Life Technologies, Gaithersburg, MD]) and centrifuged at 300*g* for 5 minutes. The cell pellets were resuspended in expansion medium and cultured in flasks. Cell yields were 5–12 million/cm^2 of skin. Medium was changed every 3–4 days. After confluence, cells were treated with 0.5% Dispase for 15 minutes, and the floating cells were discarded. After another 3 days of culture, cells from each animal were lifted using a solution containing 0.25% trypsin and 5 m*M* EDTA (Sigma, St. Louis, MO). These cells were combined and either plated to serve as the fibroblast control or purified to obtain DIAS cells.

To obtain the DIAS subpopulation, the lifted cells were seeded in a tissue culture–treated flask and allowed to attach for 10 minutes, after which the floating cells (F-DIAS) were removed. The attached cells, which represented $<10\%$ of the entire population, were washed 3 times with PBS and continued to be cultured in expansion medium for another 5 days. The cells were then harvested as DIAS cells for use in the subsequent chondroinduction process. For the monolayer portion of this study, day 0 was defined as the day that cells were to be seeded onto the aggrecan surface.

DIAS cells were chondroinduced by plating on ACS. These were prepared as previously described by French and associates (25). The concentration of aggrecan (Sigma) was 10 $\mu g/cm^2$ per 24-well plate. DIAS cells, chondrocytes, and fibroblasts were seeded on ACS at a concentration of 2×10^5 cells/well in 0.3 ml of expansion medium. After 24 hours, 1 ml of chemically defined medium (DMEM containing 1% penicillin–streptomycin–amphotericin B, 1% nonessential amino acids, 10 ng/ml transforming growth factor $\beta 1$ [PeproTech, Rocky Hill, NJ], 100 ng/ml recombinant human insulin-like growth factor [PeproTech], $10^{-7}M$ dexamethasone [Sigma], 50 μg/ml ascorbic acid-2-phosphate [Acros Organics, Geel, Belgium], 0.4 m*M* proline [Acros Organics], and 50 mg/ml ITS+ Premix [BD Biosciences, Bedford, MA]) was changed in each well to reach a final volume of 1 ml, and the medium was changed every 2 days for 2 weeks. As positive controls, goat articular cartilage chondrocytes were obtained as previously described (27).

Table 1. Primers and probe sequences used in real-time polymerase chain reaction analysis

Name	Primers (5′ to 3′) and probe sequences (5′ to 3′)	GenBank accession no.	Product size
GAPDH		U85042	86 bp
Forward	ACCCTCAAGATTGTCAGCAA		
Reverse	ACGATGCCAAAGTGGTCA		
Probe	CCTCCTGCACCACCAACTGCTT		
Type I collagen		NM_174520	97 bp
Forward	CATTAGGGGTCACAATGGTC		
Reverse	TGGAGTTCCATTTTCACCAG		
Probe	ATGGATTTGAAGGGACAGCCTGGT		
Type II collagen		X02420	69 bp
Forward	AACGGTGGCTTCCACTTC		
Reverse	GCAGGAAGGTCATCTGGA		
Probe	ATGACAACCTGGCTCCCAACACC		
Aggrecan		U76615	76 bp
Forward	GCTACCCTGACCCTTCATC		
Reverse	AAGCTTTCTGGGATGTCCAC		
Probe	TGACGCCATCTGCTACACAGGTGA		

Chondroinduction effects of ACS on DIAS cells in monolayer culture. Triplicate samples from each cell group were collected at 24 hours, 1 week, and 2 weeks and assessed for chondrocyte-specific matrix using the following analyses. For chondrocytic nodule formation, samples were collected and photographed using a CoolPix 990 digital camera (Nikon, Melville, NY) mounted on an Axioplan 2 microscope (Zeiss, Oberkochen, Germany). For glycosaminoglycan (GAG) detection, Safranin O staining was performed after 10 minutes of formalin fixation. Cells were incubated with 1% acetic acid, and Safranin O was applied for 2 minutes. Cells were then photographed after a water rinse.

Type II collagen (CII) was detected using immunohistochemistry. Briefly, formalin-fixed cells were incubated with CII primary antibody (Chondrex, Redmond, WA) and detected using the Vectastain ABC kit (Vector, Burlingame, CA) according to the instructions provided. A quantitative sandwich enzyme-linked immunosorbent assay (ELISA) for CII was also performed (29), using a monoclonal capture antibody (6009) and a polyclonal detection antibody (7006) (Chondrex).

Semiquantitative reverse transcriptase–polymerase chain reaction (PCR) analyses were performed to measure the expression of type I collagen (CI), CII, cartilage oligomeric protein (COMP), and aggrecan. RNA isolated using an RNAqueous kit (Ambion, Austin, TX) was reverse-transcribed using StrataScript RT enzyme and kit (Stratagene, La Jolla, CA) at 600 ng RNA per reaction. After transcription, PCR was performed using the Rotor-Gene 3000 real-time PCR system (Corbett Life Science, Sydney, New South Wales, Australia). The real-time analysis consisted of 15 minutes at 95°C, followed by 55 cycles of 15 seconds at 95°C, and 30 seconds at 60°C. Primer and probe sequences and concentrations are shown in Table 1. The day-0 control was obtained by isolating messenger RNA (mRNA) from fibroblasts prior to seeding onto ACS.

Immunofluorescence was used to detect filamentous actin (F-actin) and vinculin. After 36 hours of culture on ACS or uncoated control surfaces, cells were rinsed with PBS, fixed in 4% paraformaldehyde, permeabilized with Triton X-100, and blocked with 1% bovine serum albumin. For vinculin visualization, cells were incubated with monoclonal anti-vinculin IgG (Sigma), followed by incubation with Alexa Fluor 488–conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). F-actin was visualized using rhodamine and phalloidin staining (Molecular Probes). Slides were viewed using an Axioplan 2 microscope with a CoolSnapHQ CCD camera (Photometrics, Tucson, AZ).

Fabrication of in vitro cartilage-like construct. Using the chondroinduction evaluation described above, 7 days was chosen as the optimal ACS exposure time for chondroinduction. Thus, chondrocytes, DIAS cells, or F-DIAS cells were plated on 24-well ACS at 2×10^5 cells/well. After 7 days, cells were harvested by scraping and were seeded to form self-assembled constructs, as previously described (27). Briefly, a silicon-positive die consisting of cylindrical prongs (3 mm diameter $\times$ 10 mm long) was used to form a 2% agarose mold. The mold was then separated from the silicon-positive die and saturated with defined medium containing 1% FBS. For each construct, cells harvested from the 24 wells were combined and suspended in 50 μl of defined medium with 1% FBS and seeded into the agarose molds. Within 24 hours, the cells formed attached constructs, and these constructs were maintained in the agarose molds for 2 weeks. Medium was changed every 2 days. For the 3-D portion of this study, day 0 was defined as the day that cells were seeded into the agarose wells.

Construct histology. After 2 weeks, constructs were collected to evaluate cartilage-specific matrix deposition, using Safranin O to determine GAG distribution and immunohistochemistry to detect CII, CI, chondroitin 4-sulfate, and chondroitin 6-sulfate.

Statistical analysis. Results are expressed as the mean $\pm$ SD. Data were assessed by 3-factor analysis of variance. P values less than 0.05 were considered significant.

RESULTS

Morphologic response of DIAS cells to ACS. The ACS used in this study resulted in the formation of nodules, similar in both size and number, in both DIAS

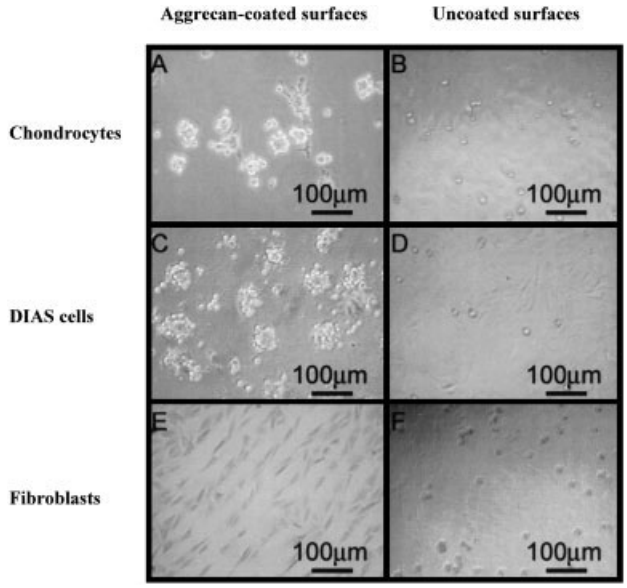

Figure 1. Aggrecan-induced morphologic changes in chondrocytes, dermis-isolated, aggrecan-sensitive (DIAS) cells, and fibroblasts after 1 day. Chondrocytes aggregated into nodules on aggrecan-coated surfaces (ACS). The round shape of peripheral cells (**A**) is characteristic of the chondrocytic phenotype. In contrast, chondrocytes spread and grew as a monolayer on the uncoated surface (**B**). DIAS cells also aggregated into nodules on ACS. The rounded appearance of peripheral cells (**C**) was similar to the finding in chondrocytes. On the uncoated surface, DIAS cells also spread and grew as a monolayer (**D**). Unlike chondrocytes or DIAS cells, fibroblasts grown on ACS were spindle shaped (**E**), and no nodules were observed. Fibroblasts also grew as a monolayer on the uncoated surface (**F**).

cells and chondrocytes. No nodules could be detected in the fibroblast group on ACS. Instead, the characteristic spindle-shaped morphology of fibroblasts was observed.

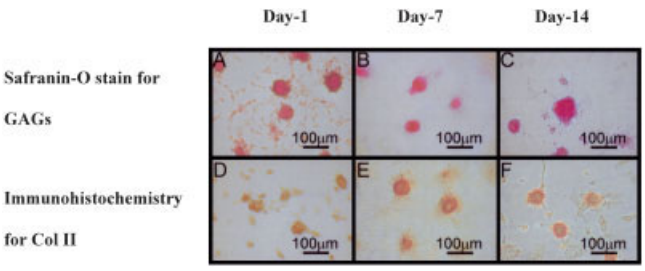

Figure 2. Detection of cartilage-specific extracellular matrix in dermis-isolated, aggrecan-sensitive cells cultured for 1–14 days on aggrecan-coated surfaces. Using Safranin O, all nodules stained positively for glycosaminoglycans (GAGs) (**A–C**). Immunohistologic staining was positive for type II collagen (Col II) (**D–F**), which is evidence of chondrocytic nodule formation. Results from uncoated controls are not shown due to negligible staining. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org

The uncoated surface resulted in monolayers for all cell types examined (Figure 1).

Chondrocyte-specific ECM detection. All nodules formed using DIAS cells on ACS stained positively for GAGs (Figures 2A–C) and for CII (Figures 2D–F). All cells grown on uncoated surfaces were negative for both stains (results not shown). The formation of nodules exhibiting GAGs and CII matrix provided evidence of chondroinduction of DIAS cells.

Quantification of cartilage-specific matrix gene expression and protein production. The effect of ACS on cartilage-specific matrix gene expression and on protein production was investigated. DIAS cells and fibroblasts were grown either on ACS or on uncoated surfaces for 14 days. Expression of mRNA for 3 positive markers of chondroinduction (aggrecan, CII, and

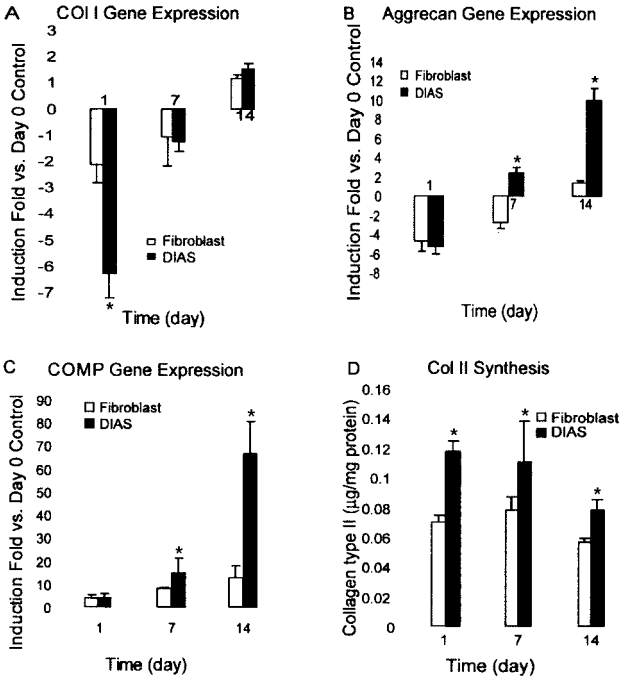

Figure 3. Expression and synthesis of cartilage-specific markers in dermis-isolated, aggrecan-sensitive (DIAS) cells compared with fibroblasts. Reverse transcriptase–polymerase chain reaction results showed significant inhibition of type I collagen (Col I) gene expression for 1–7 days in both cell populations (**A**). On aggrecan-coated surfaces (ACS), aggrecan and cartilage oligomeric protein (COMP) gene expression was significantly increased in DIAS cells compared with fibroblasts on days 7 and 14 (**B** and **C**). Enzyme-linked immunosorbent assay showed that aggrecan coating of surfaces resulted in higher levels of type II collagen in DIAS cell cultures than in fibroblast cultures (**D**) at every time point tested. These data suggest that the extent of chondroinduction undergone by DIAS cells when exposed to ACS is significantly greater than that undergone by fibroblasts. Values are the mean and SD. $* = P < 0.05$ versus fibroblasts.

COMP) and 1 negative marker of chondroinduction (CI) was measured. In addition, ELISA was used to determine the actual protein synthesis level of CII.

After exposure to ACS, expression of CI immediately decreased in both DIAS cells and fibroblasts, although this suppression was initially more pronounced in DIAS cells. This suppression did not persist beyond 7 days (Figure 3A).

By comparing the expression and synthesis of cartilage-specific markers, DIAS cells were shown to possess a greater chondroinduction potential compared with fibroblasts (Figure 3). Specifically, after seeding onto ACS, aggrecan gene expression in DIAS cells was significantly higher ($P < 0.05$) than that in fibroblasts at 7 and 14 days (Figure 3B). Similarly, COMP expression by DIAS cells was also significantly higher ($P < 0.05$) than that in fibroblasts (Figure 3C) at 7 and 14 days. By day 14, COMP expression in DIAS cells was 5-fold higher than in fibroblasts. More important, protein synthesis levels of CII (Figure 3D), another cartilage-specific marker, were found to mirror COL2 gene expression (data not shown) and were significantly higher ($P < 0.05$) at all time points in DIAS cell populations when compared with fibroblasts (Figure 3D).

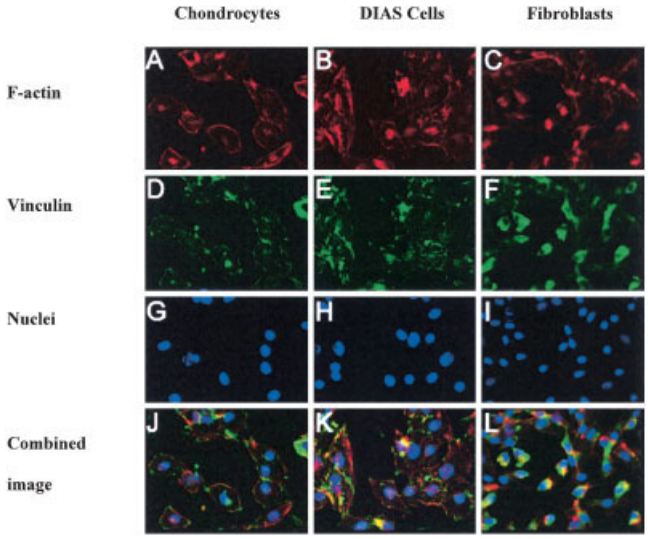

Figure 4. Reorganization of filamentous actin (F-actin) and vinculin in chondrocytes, dermis-isolated, aggrecan-sensitive (DIAS) cells, and fibroblasts after 36 hours of monolayer culture on aggrecan-coated surfaces. F-actin was stained with rhodamine and phalloidin (red) (**A–C**). Vinculin was stained with Alexa Fluor 488 (green) (**D–F**). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue) (**G–I**). A punctated distribution of F-actin was seen at the periphery of chondrocytes (**A**) and DIAS cells (**B**), while a dense collection of F-actin was seen throughout the fibroblasts (**C**). The organization of vinculin mirrored that of F-actin in each group. Combined images with all 3 stains were also created (**J–L**). On uncoated control surfaces, the 3 cell groups exhibited similar F-actin and vinculin distribution (results not shown). (Original magnification × 63.)

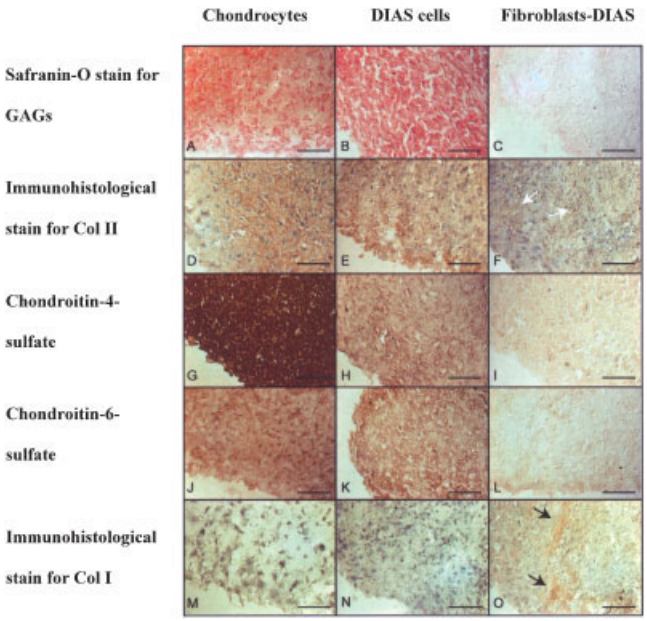

Figure 5. Detection of cartilage-specific extracellular matrix (ECM) in constructs self-assembled for 2 weeks using chondrocytes, dermis-isolated, aggrecan-sensitive (DIAS) cells, and floating DIAS (F-DIAS) cells. Sections taken from chondrocyte constructs were stained for glycosaminoglycans (GAGs) (**A**), type II collagen (Col II) (**D**), chondroitin 4-sulfate (**G**), chondroitin 6-sulfate (**J**), and type I collagen (**M**). Spherical chondrocytes were noted within a matrix containing GAGs, type II collagen, chondroitin 4-sulfate, and chondroitin 6-sulfate, indicative of cartilage formation. DIAS construct also stained positively for the same cartilage-specific ECM (**B, E, H,** and **K**). Type I collagen was not observed within chondrocyte or DIAS constructs (**M** and **N**). In contrast, constructs from F-DIAS cells exhibited negligible GAG staining (**C**), poor type II collagen staining (**F**) (**arrows**), poor chondroitin 4-sulfate staining (**I**), and negligible chondroitin 6-sulfate staining (**L**), while staining for type I collagen (**O**) (**arrows**) was observed. Bars = 50 μm.

Initiation of chondroinduction by fluorescence imaging of cytoskeletal organization of ACS. Since cells adhere to the extracellular substratum by focal adhesion, we investigated whether ACS had any effect on this event. After 36 hours in culture, cells were labeled with phalloidin and rhodamine, which specifically bind to the F-actin cytoskeleton, and with antivinculin antibodies. Differences were observed among cell groups cultured on ACS (Figure 4), but not among cells cultured on uncoated surfaces (results not shown). Fibroblasts seeded on ACS formed strong polarized F-actin fiber bundles distributed throughout the cytoplasm, accompa-

nied by abundant stress fibers (Figure 4C). In contrast, the formation of F-actin fiber bundles was significantly inhibited in both chondrocytes and DIAS cells (Figures 4A and B). In these cells, F-actin was preferentially lost from the central cytoplasm and became concentrated at the cell periphery. Treatment with antivinculin antibodies revealed that the distribution of vinculin in each cell mirrored F-actin distribution (Figures 4D–F).

Histologic evaluation of engineered constructs. Cells in all groups aggregated and formed constructs in vitro, 2 weeks after self-assembly. Samples from each group were then collected and sectioned for histologic evaluation. Histologic and immunohistochemical studies in cartilage ECM from DIAS constructs revealed strong and even staining for GAGs, CII, chondroitin 4-sulfate, and chondroitin 6-sulfate (Figures 5B, E, H, and K). In contrast, the F-DIAS groups stained poorly for all the above-mentioned cartilage components (Figures 5C, F, I, L, and O). CI was not observed in either the chondrocyte or the DIAS constructs, while colonies of cells positive for CI (Figure 5O, arrows) were detected in F-DIAS groups. This, in combination with the observation that a trace amount of CII was localized in colonies within F-DIAS cells (Figure 5F, arrows), implies that complex heterogeneous cell populations exist within the F-DIAS constructs in terms of their chondroinduction potential.

DISCUSSION

Previous studies using dermal fibroblasts showed that demineralized bone powder could induce the formation of colonies exhibiting a chondrocytic phenotype. However, no further evidence exists to show whether these chondroinduced cells can be considered to originate from stem cells, fully mature fibroblasts, or a dermal subpopulation of cells with latent chondrogenic potential (17). Although a number of researchers have investigated techniques to isolate subpopulations from the dermis for different purposes (15,30), none of these subpopulations has been isolated specifically for cartilage regeneration. Thus, there is an absence of well-defined and efficient protocols for the selective isolation and proliferation of dermis-derived cells, followed by directing their differentiation into the chondrogenic lineage in vitro.

This study demonstrates that a specific subpopulation of dermis-derived cells could be isolated from goat skin by exploiting their rapid adherence to tissue culture polystyrene surfaces, and that these cells (DIAS cells) have the potential for ACS-facilitated chondroinduction, as evidenced by the production of rich, cartilage-specific ECM (Figure 2), high levels of cartilage-specific gene expression (Figure 3), and F-actin and vinculin distribution similar to that in chondrocytes (Figure 4). Tissue-engineered constructs formed by DIAS cells exhibited cartilage-specific ECM components throughout (Figure 5), while constructs formed using other dermis-derived subpopulations resulted in heterogeneous matrices. Thus, this study demonstrates the development of a protocol to form uniform tissue-engineered cartilage constructs from a chondroinduced, purified dermis-derived subpopulation. This protocol reduces the likelihood of heterogeneous cell subpopulations spontaneously differentiating into divergent lineages and, in the case of fibroblasts, decreases the risk of fibrochondrocytic formation.

A rapid adherence process has previously been used to isolate stem cells from the skin epidermis (31) and from adipose tissue (32). In this study, we developed a modified rapid adherence process to isolate DIAS cells from goat dermis for chondroinduction. Instead of selecting all rapidly adhering cells from the dermis, the Dispase-sensitive subpopulations are first removed (since these populations also contain rapidly adhering cells). Rapidly adhering cells from the remaining subpopulations are then isolated based on their adherence time. Cells that adhered to the plastic surface within 10 minutes were chosen because they produced the highest nodule numbers when seeded on ACS compared with cells from other time points (data not shown).

The purified cells, termed DIAS cells, are dermis-isolated, aggrecan-sensitive cells, which, when cultured on ACS, express and produce cartilage-specific matrices. This method allows for a high-throughput approach to collect chondroinducible cultures. DIAS cells were chondroinduced when seeded on ACS, and were phenotypically, morphologically, and functionally similar to chondrocytes. In situ activity of DIAS cells might be suppressed in the in vivo microenvironment through signaling from skin ECM and/or from mature fibroblasts. However, in vitro or ectopically, the chondroinduction process may be initiated due to the presence of an enriched environment of DIAS cells and/or exposure to aggrecan or other cartilage-specific ECM components.

Aggrecan has been found to play an essential role in the chondrogenesis process and the subsequent maintenance of the chondrocyte phenotype in vivo (33,34) and in vitro (35). Based on these observations, it was thus of interest in the present study to observe the effects of ACS on DIAS cell chondroinduction in terms

of cell morphology, ECM gene expression and production, and cytoskeleton organization.

Chondrocytes, DIAS cells, and fibroblasts were seeded on ACS in this study. Fibroblasts showed a spindle-like morphology on ACS 24 hours after seeding (Figure 1E). However, consistent with previous research (35), we found that chondrocytes responded sensitively to ACS by organizing into nodules, suggesting the presence of a different interacting pathway between chondrocytes and fibroblasts. DIAS cells used an aggrecan-sensitive pathway significantly different from that of fibroblasts. However, DIAS cells formed nodules similar in size and number to those in chondrocytes on ACS (Figures 1A and C), suggesting that analogous early-stage cell–matrix interaction mechanisms may exist between DIAS cells and chondrocytes when cultured on ACS.

Consistent with the morphologic findings, the ECM results also show that DIAS cells have a higher potential for chondroinduction compared with unpurified, heterogeneous fibroblast subpopulations. Throughout the entire experimental period, nodules formed by DIAS cells seeded on ACS were shown to stain positively for Safranin O (Figures 2A–C) and for CII (Figures 2D–F). In contrast, both DIAS and fibroblast cells seeded on uncoated surfaces showed negative staining for both GAG and CII under the same conditions (results not shown), which is common for dermis-derived cells. DIAS cells exposed to ACS expressed cartilage marker genes more rapidly and more potently than did fibroblasts (Figures 3B and C). Moreover, ACS appeared to inhibit the fibroblastic phenotype in DIAS cells, as evidenced by significant inhibition of COL1 gene expression at 1 day and 7 days (Figure 3A).

However, it was also observed that COL1 gene expression recovered with time in each cell group, and, since higher levels of expression of other cartilage-specific markers were seen from 7 days onward, 7 days was chosen as the transition between monolayer and 3-D culture. Compared with 3-D culture, 2-dimensional (2-D) surfaces appeared less optimal for chondroinduction. This was confirmed by immunohistochemistry of 3-D cultures. Indeed, CI was not observed in self-assembled DIAS constructs (Figure 5N), while cartilage-specific markers were retained (Figures 5B, E, H, and K). Taken together, these findings confirmed that DIAS cells have higher chondroinduction potential than fibroblasts when exposed to ACS.

The influence of substrate on morphogenesis depends on cell type as well as cellular properties such as cytoskeletal organization, cell adhesion, and cell–cell interactions (36). To further our understanding of the regulatory mechanisms of aggrecan, chondrocytes, DIAS cells, and fibroblasts were cultured on ACS for 36 hours. Chondrocytes and DIAS cells were found to organize their F-actin on ACS in a similar pattern, which was significantly different from that of fibroblasts. Fewer stress fibers were found in DIAS cells and chondrocytes than in fibroblasts. Furthermore, the distribution of vinculin in each group mirrored its F-actin distribution (Figure 4). The observed F-actin patterns of DIAS cells and chondrocytes in this study were similar to those reported for chondrocytes in monolayer (37,38). This implies that the 2 cell types have similar cell–matrix interactions.

Studies of a number of cell types have shown that F-actin organization plays an important role in a large number of cellular events, including shape alteration (39), cell signaling (40), secretion (41), and ECM assembly (42). Any one or a combination of the above-described events may thus be precipitated by the F-actin organization brought about by cell–matrix interactions. Indeed, chondrocytes were found to respond to ECM components, including hyaluronic acid (43) and CI (23), by reorganizing their F-actin in vitro, resulting in the regulation of various chondrocyte behaviors such as cell shape determination, chondrogenesis initiation, chondrocytic phenotype maintenance, and chondrocyte hypertrophy (44,45). Again, any one or a combination of these events may have occurred as chondrocytes were seeded onto ACS. In this study, specific cell–matrix interactions led to F-actin and vinculin reorganization. This reorganization may have resulted in the subsequent changes in various DIAS cell events that ultimately led to chondrogenic phenotype formation of these cells in 2-D. These specific cell–matrix interactions may also lead to a temporal and spatial self-assembly process in 3-D.

The assembly of cells into functional multicellular organisms in 3 dimensions involves F-actins, the primary sites at which cells detect and adhere to their ECM (46). Points of F-actin and vinculin colocalization have been shown to be sites where chondrocytes adhere to the articular cartilage ECM (47). Many researchers have chosen to focus on the 3-D study of cell-seeded matrices in an effort to engineer articular cartilage in vitro for implantation in vivo (7,48). For these purposes, we have recently developed a self-assembly process (27). By using this scaffoldless approach with chondrocytes, we have successfully obtained cartilage-like constructs that mimic native cartilage in terms of biochemical and biomechanical properties. Although the exact mecha-

nisms of the self-assembly process initiated and accomplished by chondrocytes are not known, temporal and spatial interactions between the chondrocytes and their ECM environments have been suggested to be essential for successful cartilage development, based on in vivo and in vitro research, as previously reviewed (49). Encouraged by these findings, we were motivated to investigate whether the chondroinduced DIAS cells in 2-D culture could undergo a self-assembly process similar to the one chondrocytes underwent in a 3-D environment.

When chondroinduced DIAS cells were seeded in agarose molds, they aggregated and self-assembled into cartilage-like constructs, as expected. Two weeks after seeding, the constructs were sectioned for cartilage-specific ECM detection. Similar to constructs formed by chondrocytes, high levels of total GAG, CII, chondroitin 4-sulfate, and chondroitin 6-sulfate were found in DIAS constructs (Figures 5A, B, D, E, G, H, J, and K), which indicated cartilage formation. In contrast, F-DIAS cell constructs showed poor staining for all of the above-mentioned cartilage-specific matrices; instead, colonies of cells that stained positively for CI were detected. Furthermore, compared with the homogeneous distribution of cartilage-specific ECM in DIAS constructs, colonies of cells that stained positively for CI (Figure 5O, arrows) and CII (Figure 5F, arrows) showed an uneven distribution of different dermis-derived subpopulations in the F-DIAS constructs. This further supports our hypothesis that subpopulations of dermis-derived cells must first be purified, in order to obtain cells that can undergo chondroinduction in a uniform manner.

Differences in ECM levels between chondrocyte constructs and DIAS constructs still exist. This may be remedied by optimizing the protocol to use different adhesion times to select for DIAS cells with higher chondroinduction potential. In addition to ACS, optimized combinations of growth factors might be important in chondroinduction and the subsequent self-assembly of the DIAS cells.

Dermis-derived cells are attractive since they provide autologous cells without causing complications at the donor site, due to the high regenerative capacity of skin (50). These cells can also be harvested with a low degree of invasiveness. As we search for new paradigms for clinical intervention in pathologic cartilage conditions, the results of this study suggest that DIAS cells may be viable candidates for various cartilage repair strategies. The 3-step process developed in this study may contribute to a potential therapeutic strategy that uses the self-assembly of chondroinduced DIAS cells to produce cartilaginous tissue in vitro, which translates to an autologous transplant in vivo. Since this is the first study in which a cell subpopulation that is sensitive to cartilage-specific ECM has been isolated and chondroinduced from adult dermis, much work is clearly still needed to fully elucidate the mechanism of the chondroinduction process. Such work may include the use of biologic markers of skin-derived stem cells to trace the potential origin of these cells.

AUTHOR CONTRIBUTIONS

Dr. Deng had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Drs. Deng, Hu, and Athanasiou.
Acquisition of data. Dr. Deng.
Analysis and interpretation of data. Drs. Deng, Hu, and Athanasiou.
Manuscript preparation. Drs. Deng, Hu, and Athanasiou.
Statistical analysis. Drs. Deng, Hu, and Athanasiou.

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