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METHODS ARTICLE

Adult Dermal Stem Cells for Scaffold-Free Cartilage Tissue Engineering: Exploration of Strategies

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Dermis-isolated adult stem (DIAS) cells, abundantly available, are attractive for regenerative medicine. Strategies have been devised to isolate and to chondroinduce DIAS cells from various animals. This study aimed to characterize DIAS cells from human abdominal skin (human dermis-isolated adult stem [hDIAS] cells) and to compare and to refine various chondroinduction regimens to form functional neocartilage constructs. The stemness of hDIAS cells was verified (Phase I), three chondroinduction pretreatments were compared (Phase II), and, from these, one regimen was carried forward for refinement in Phase III for improving the mechanical properties of hDIAS cell-derived constructs. Multilineage differentiation and mesenchymal stem cell markers were observed. Among various chondroinduction pretreatments, the nodule formation pretreatment yielded constructs at least 72% larger in diameter, with higher glycosaminoglycan (GAG) content by 44%, compared with other pretreatments. Furthermore, it was found that culturing cells on nontissue culture-treated surfaces yielded constructs (1) on par with constructs derived from aggrecan-coated surfaces and (2) with superior mechanical properties than constructs derived from cells cultured on tissue culture-treated surfaces. After the nodule formation pretreatment, combined supplementation of TGF-β1, IGF-I, and fetal bovine serum significantly enhanced aggregate modulus and shear modulus by 75% and 69%, respectively, over the supplementation by TGF-β1 alone. In summary, human skin-derived DIAS cells are responsive to chondroinduction for forming neocartilage. Furthermore, the mechanical properties of the resultant human constructs can be improved by treatments shown to be efficacious in animal models. Advances made toward tissue-engineering cartilage using animal cells were shown to be applicable to hDIAS cells for cartilage repair and regeneration.

Keywords: dermal mesenchymal stem cells, cartilage tissue engineering, self-assembly, substrate in cartilage engineering, nodule formation

Impact Statement

Abundance and the relative ease in the harvesting of autologous dermal stem cells render them an attractive cell source for regenerative medicine. This study sought to investigate the potential use of human dermis-isolated adult stem (hDIAS) cells as an alternative cell source for cartilage tissue engineering. Results indicate that hDIAS cells express stem cell markers, possess multilineage differentiation potential including the chondrogenic lineage, and respond positively to chondroinductive stimulus that improves neocartilage functional properties. These data suggest that hDIAS cells can serve as a potential alternative cell source for cartilage repair and regeneration.

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Introduction

HUMAN ADULT MESENCHYMAL stem cells (MSCs) have been extensively explored for tissue engineering due to their abundance and multipotency. Human skin is one of the most abundant sources of MSCs. Skin-derived stem cells are appealing for therapeutic purposes because they are easily accessible, can be autologous, and, for nonhomologous uses, are typically not affected by the disease that necessitates their use.

Dermis has been shown to contain MSCs capable of differentiating into at least three mesenchymal lineages.[1–5] Stem cells derived from the dermis were also able to differentiate toward ectodermal[2,6] and endodermal[7] lineages. For example, skin-derived precursors isolated from human neonatal foreskin can differentiate into neural[8] and mesodermal lineages, including the chondrogenic lineage.[9] Given the accessibility and abundance of adult dermal tissue, dermis-derived stem cells are attractive for tissue engineering applications.

Cartilage tissue engineering has potential for cartilage repair. Osteoarthritis (OA) remains a major health problem worldwide[10,11] and affects over 30 million people in the United States alone.[12] Current cartilage repair therapies do not prevent OA. Human articular chondrocytes are currently used clinically and in the development of tissue-engineered cartilage repair products.[13] However, due to their limited availability, chondrocytes are expanded *in vitro*, causing dedifferentiation and loss of chondrogenic properties.[14] Repair tissues resulting from inappropriate phenotypes exhibit inferior mechanical properties that are unsuitable for durable repair.

Given that MSCs can endure immense cell expansion and still differentiate into chondrocytes, adult MSCs have been continually explored as an alternative cell source in cartilage tissue engineering. Adult stem cells in the dermis can be isolated via rapid adherence to tissue culture-treated plates.[15] These selected cells, termed dermis-isolated adult stem (DIAS) cells, have shown promise for engineering neocartilage.[15,16]

Toward the use of DIAS cells for cartilage engineering, methods of expansion, chondroinduction, and neotissue generation have been explored using animal-derived cells.[15,16] DIAS cells isolated from caprine abdominal skin have been shown to form large numbers of chondrocytic nodules on surfaces coated with exogenously derived aggrecan (hereon referred to as aggrecan-coated surfaces, or ACS), and the cells within these nodules exhibited high aggrecan and collagen II gene expression.[15] Self-assembled constructs formed by these chondroinduced DIAS cells contained comparable levels of staining for glycosaminoglycan (GAG) and type II collagen to caprine chondrocyte-derived constructs.[15]

Caprine DIAS cells have been shown to exhibit multilineage differentiation and responded to chondrogenic stimuli, yielding constructs with improved biochemical and mechanical properties.[16] Neocartilage has also been formed using human DIAS (hDIAS) cells from different anatomical locations.[17] However, it remains unclear whether hDIAS cells express stem cell markers, have multilineage differentiation potency, and whether hDIAS cells respond to chondrogenic stimuli similarly to animal DIAS cells.

The objectives of this study are to elucidate the nature of hDIAS cells through interrogation of multilineage differentiation and common stem cell markers and to compare and refine cartilage tissue engineering regimens.[15–17] This study was conducted in three phases. In Phase I, the multilineage differentiation potential of hDIAS cells and their expression of stem cell markers were examined. It was hypothesized that hDIAS cells would differentiate into three mesenchymal lineages and that these cells would express common MSC markers. In Phase II, three chondroinductive pretreatments were compared to select one for further optimization. The hypothesis was that different chondroinductive pretreatments would influence the ability of hDIAS cells to form constructs.

Carrying forward the regimen that yields the most biomimetic neocartilage from Phase II, Phase III evaluated how substrate and growth factor combinations affected the functional properties of engineered constructs. It was hypothesized that refining nodule formation on ACS would improve the chondrogenic potential of hDIAS cells and the functional properties of constructs. It was also hypothesized that applying a growth factor combination efficacious in animal models to hDIAS cells would improve construct mechanical properties.

Methods

hDIAS cells were used. This study was conducted in three phases; in Phase I, the multilineage differentiation potential of hDIAS cells and their expression of stem cell markers were examined; in Phase II, three chondroinductive, pretreatment regimens were compared to select one pretreatment regimen for further optimization; and in Phase III, the effects of various substrates and growth factor combinations on the mechanical properties of engineered constructs were evaluated (Fig. 1). This study focused on cells from a single donor to eliminate potential individual variability. A study comparing the chondrogenic potential of hDIAS cells from abdominal skin between different donors was conducted by our groups previously.[17]

Experiment

Cell isolation

Cells were isolated from the abdominal skin of a 49-year-old female under an institutional review board exemption. Tissue was obtained from the University of California, Davis (UC Davis) Medical Center as waste from a surgery unrelated to this study. The adipose tissue underlying the dermis was removed and discarded. The epidermis and dermis were separated via 0.2% dispase II (Roche) digestion overnight at 4°C. The dermis was then minced and digested in 0.25% pronase (Sigma-Aldrich) containing 3% fetal bovine serum (FBS; Atlanta Biologicals) at 37°C for 1 h, followed by digestion in a 0.2% w/v collagenase type II (Worthington) solution containing 2% FBS at 37°C for 16–18 h.

Dermal cells were filtered through 70 μm strainers, washed two to three times with high-glucose Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX™ (Thermo Fisher Scientific) and 1% penicillin/streptomycin/fungizone (P/S/F; Lonza), and cryopreserved in liquid nitrogen. Dermal cells were thawed and seeded in monolayer in DMEM containing 10% FBS, 1% P/S/F, and 1% MEM nonessential amino acids mix (MEM NEAA; Thermo Fisher Scientific). At over 95% confluence, cells were detached using 0.05% trypsin-EDTA (Thermo Fisher Scientific), passaged, and allowed to adhere for 10 min. Nonadhered cells were removed via three consecutive rinses. The remaining adherent cells (rapidly adhered cells) were defined as hDIAS cells (Fig. 1).

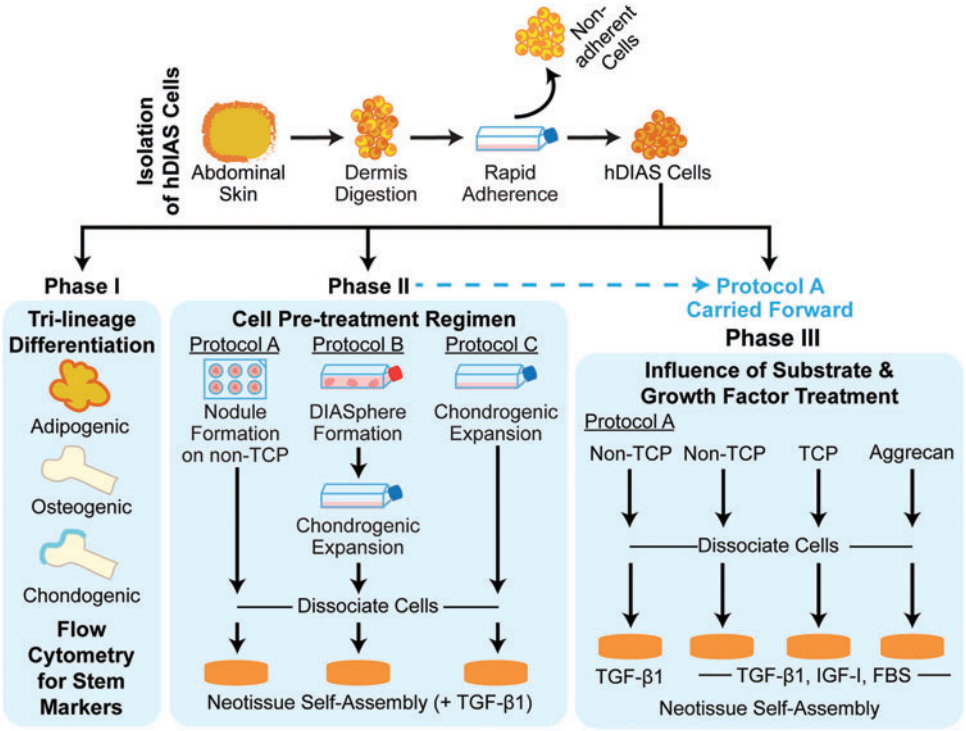

FIG. 1. Schematic layout of the performed experiments. In Phase I, trilineage differentiation and expression of stem cells markers was validated. In Phase II, three chondroinductive pretreatment regimens were explored with the intention to select one regimen that would generate cells able to yield cartilage constructs with most improved biochemical and mechanical properties. Carrying forward the selected regimen, in Phase III, assessed were the effects of various substrates and growth factors on improving mechanical properties of tissue-engineered cartilage. Color images are available online.

Phase I

To evaluate multilineage potential, hDIAS cells were grown in adipogenic, osteogenic, or chondrogenic culture conditions (Fig. 1).[18] For adipogenic and osteogenic differentiation, 1.5×10^4 cells/well were plated in a 24-well plate. Adipogenic differentiation medium contained DMEM with high glucose, 5% FBS, 1% P/S/F, 1% MEM NEAA, 1 µM dexamethasone (Sigma-Aldrich), 0.5 mM isobutyl methylxanthine (Sigma-Aldrich), and 0.2 mM indomethacin (Sigma-Aldrich). Osteogenic differentiation medium contained DMEM with high glucose, 10% FBS, 1% P/S/F, 1% MEM NEAA, 100 nM dexamethasone, 10 mM β-glycerophosphate (Sigma-Aldrich), 250 mM ascorbate-2-phosphate (Sigma-Aldrich), and 50 ng/mL BMP-2 (Peprotech).

For chondrogenic differentiation, 250,000 cells were added to a round-bottomed polystyrene 96-well plate (Costar 3799; Corning) in 0.2 mL of chondrogenic medium (CHG; DMEM with high glucose, containing 1% P/S/F, 1% ITS+ premix, 1% MEM NEAA, 100 nM dexamethasone [Sigma-Aldrich], 50 µg/mL ascorbate-2-phosphaste [Sigma-Aldrich], 40 µg/mL L-proline [Sigma-Aldrich], and 100 µg/mL sodium pyruvate [Sigma-Aldrich]), supplemented with 50 ng/mL BMP-2, and 10 ng/mL TGF-β1 (Peprotech), and centrifuged at 500 *g* for 5 min to form cell pellets.[19] All groups were maintained at 37°C and 5% CO_2 for 4 weeks, with media exchanged every other day. Samples for each condition were prepared and analyzed in triplicate.

MSC markers[20] were assessed via labeling with anti-human CD45 (clone 2D1; BD Biosciences), CD31 (clone WM 59; BD Biosciences), CD73 (clone AD2; BD Biosciences), CD90 (clone 5E10; BD Biosciences), CD105 (clone 255; BD Biosciences), CD146 (clone 541-10B2; Miltenyi Biotech), and CD271 (clone ME20.4; Biolegend) antibodies conjugated to distinct fluorophores. A fixable viability fluorescent dye (LIVE/DEAD™ Fixable Aqua Dead Stain Kit; Thermo Fisher Scientific) was applied. Following labeling, multicolor flow cytometry was performed on fixed cells using LSR II (BD Biosciences). From a total of 50,000 events, dead cells, doublets, and CD45- and CD31-positive cells (leukocytes and endothelial cells) were excluded. Using gating on the CD45- and CD31-negative cells, percentages of CD73-, CD90-, CD105-positive cells were recorded to determine cells with MSC markers. CD146 and CD271 were used to assess pericyte and follicle origin, respectively. Fluorescence minus one and compensation beads were used as controls. Compensation and analyses were executed on FlowJo (BD Biosciences).

Phase II

Three chondroinductive pretreatment protocols were compared: (1) Nodule formation protocol, (2) DIASphere protocol, and (3) Chondrogenic expansion protocol (Fig. 1). Pretreatment regimen for each protocol was applied and adapted from the previously published regimens.[15–17]

A Nodule formation protocol (Fig. 1, Protocol A) consisted of chondroinducing hDIAS cells in the form of nodules, followed by construct formation using the resultant cells.[15] Briefly, hDIAS cells were plated on nontissue culture-treated plastic (non-TCP) six-well plates (Falcon) at 1×10^6 cells per well ($\sim 1 \times 10^5$ cells/cm^2). The cells formed nodules on the non-TCP plates and were cultured for a week in CHG with 10 ng/mL TGF-β1 and 100 ng/mL IGF-I (Peprotech). The nodules were dissociated with 0.25% trypsin-EDTA for 10 min, followed by digestion in a 0.2% w/v collagenase type II solution for 30 min. The resultant cells were then seeded into constructs via the self-assembling process.[21]

A DIASphere protocol (Fig. 1, Protocol B) selected cells capable of forming multicellular spheres using a published

protocol[4]; 5×10^4 cells/mL hDIAS cells were seeded into tissue culture flasks in 3:1 low-glucose (1 g/L) DMEM and F12 (Thermo Fisher Scientific), 1% P/S/F, 20 ng/mL EGF (Peprotech), 40 ng/mL FGF-2 (Peprotech), and 2% B27 (Thermo Fisher Scientific), and allowed to form spheres. After 3–4 weeks of culture, the resultant spheres were collected and dissociated using 0.05% trypsin-EDTA and further expanded in CHG supplemented with 10% FBS and 5 ng/mL FGF-2.[17] Cells were lifted using 0.05% trypsin-EDTA and seeded into constructs via the self-assembling process.[21]

A Chondrogenic expansion protocol (Fig. 1, Protocol C) consisted of hDIAS cell expansion in monolayer in CHG, followed by construct formation using the expanded cells.[16] hDIAS cells were seeded into TCP flasks at 10,000 cells/cm^2 and grown to 80–90% confluence in CHG supplemented with 10% FBS, and 10 ng/mL FGF-2. Cells were lifted using 0.05% trypsin-EDTA and passaged three times to get the desired number of cells. The resulting cells were seeded into constructs via the self-assembling process.[21]

To form self-assembled constructs after each of the above protocols (i.e., Protocol A—Nodule formation, Protocol B—DIASphere, and Protocol C—Chondrogenic expansion), 2×10^6 cells were seeded into 3-mm-diameter agarose wells composed of 2% w/v agarose.[21] Constructs were maintained in CHG with 10 ng/mL TGF-β1. During the 2nd and 4th week of culture, 1 g porous, stainless steel posts were applied onto each construct. Medium was changed every day for the 1st week and every other day for the rest of the culture period. Four to six constructs were prepared for each protocol group. After 4 weeks of culture, constructs were collected and assessed grossly, histologically, mechanically, and biochemically.

For gross and histological assessment, in Phase I, hDIAS cells and three-dimensional (3D) pellets were fixed in 10% neutral buffered formalin (NBF), and 3D pellets were embedded in paraffin and sectioned at 5 μm. All samples were stained with Oil red O for adipogenesis, Alizarin red for osteogenesis, and Alcian blue/nuclear fast red (pH = 1) for chondrogenesis.[22] In Phase II, constructs were fixed in 10% NBF, embedded in paraffin, and sectioned at 5 μm. Slides were stained with hematoxylin and eosin as well as Alcian blue/nuclear fast red for sulfated glycosaminoglycans (sGAGs).[22]

In Phase III, constructs were cryoembedded in Histoprep (Thermo Fisher Scientific), sectioned at 10 μm, fixed in acetone, and stained with Toluidine blue. To detect type II collagen in hDIAS constructs, immunohistochemistry (IHC) was used. IHC was performed using rabbit anti-human collagen type II polyclonal antibody (AB34712; Abcam) and the Vectastain ABC-HRP kit, followed by color development using NovaRED (Phase II) or DAB (Phase III) substrate kits (Vector Laboratories, Inc.).

For biochemical and mechanical tests, the wet and dry weights of each sample were measured before and after 48 h of lyophilization, respectively. Samples were digested in 125 μg/mL papain (Sigma-Aldrich) in 50 mM phosphate buffer (pH 6.5) containing 2 mM N-acetyl cysteine (Sigma-Aldrich) and 2 mM EDTA for 18 h at 60°C. Sulfated GAGs were quantified using a dimethylmethylene blue assay (Biocolor Ltd.).

For mechanical evaluation, creep indentation was performed.[23] Construct thickness was recorded and a 0.25 g (0.0025 N) mass was applied through a 0.45-mm-radius flat, porous indenter tip. Aggregate modulus, shear modulus, and permeability were obtained by a semianalytical, seminumerical, linear biphasic model and finite element analysis.[24]

Phase III

A total of 1×10^6 hDIAS cells were seeded on non-TCP six-well plates (Falcon; Corning), TCP six-well plates (Falcon; Corning), or ACS, and cultured in CHG with 10 ng/mL TGF-β1 and 100 ng/mL IGF-I for a week. ACS coating was obtained by covering the surface of a 35 mm cell culture dish (CELLSTAR; Greiner Bio-One) with 150 μg of aggrecan, derived from bovine articular cartilage (Catalogue No. A1960; Sigma-Aldrich), dissolved in 400 μL of water, and allowing the aggrecan solution to evaporate for 4 h in a tissue culture hood.

After a week of culture, individual cells were obtained and seeded into constructs using the self-assembling process. To examine the effect of growth factors during self-assembly, constructs were cultured either in CHG with 10 ng/mL TGF-β1 only, or CHG supplemented with 10 ng/mL TGF-β1, 100 ng/mL IGF-I, and 1% FBS (the treatment previously used in animal models). Three to four constructs were prepared for each group. Constructs were evaluated histologically and mechanically, as per previous phases.

Statistics

All mechanical and biochemical data were analyzed using $n = 4$–6 constructs (Phase II) and $n = 3$–4 (Phase III) per group. All data are shown as mean ± standard deviation. For comparison between two groups, a Student's *t*-test was performed. Significant differences are indicated by an asterisk. Statistical differences of three or more groups were analyzed using one-way analysis of variance with Tukey's *post hoc* test ($p < 0.05$) (JMP12). Statistical significance is indicated by bars not sharing the same letter.

Results

Phase I: multilineage differentiation potential and stemness of hDIAS cells

Multilineage differentiation and the expression of MSC markers were observed for hDIAS cells (Figs. 2 and 3). hDIAS cells differentiated into osteogenic, adipogenic, and chondrogenic lineages (Fig. 2). Before differentiation, hDIAS cells expressed MSC markers CD73, CD90, and CD105 (Fig. 3). Approximately 57% of these cells were also CD146 positive, and <2% were CD271 positive. Although CD146 and CD271 are not routinely considered stem cell markers, these epitopes have been described as stem markers of dermal cells.[5,25]

Phase II: comparison of pretreatments

Constructs were first evaluated by grossly and then by the primary design criterion of mechanics. Any treatment that did not yield mechanically testable constructs was not subjected to mechanical or biochemical evaluation. Construct gross morphology varied with pretreatment regimen. Not all seeded cells were incorporated into constructs. In all groups, a loose and dispersed population of cells was observed adjacent to

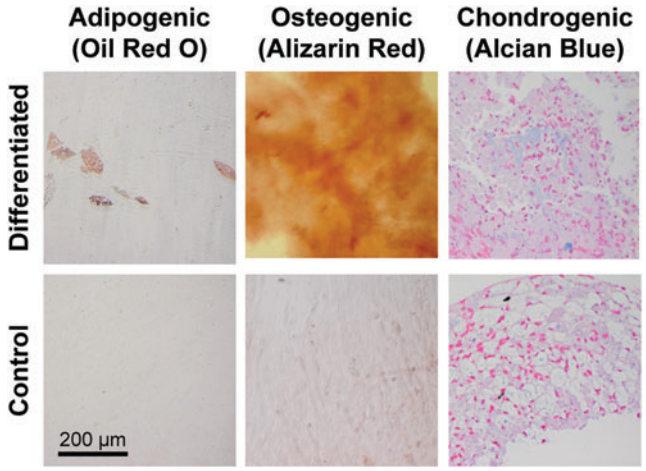

FIG. 2. Trilineage differentiation and stem cell marker expression of hDIAS cells. hDIAS cells were cultured in adipogenic, osteogenic, and chondrogenic differentiation media for 4 weeks. *Left*: Oil red O staining for adipogenesis. *Middle*: Alizarin red staining for osteogenesis. *Right*: Alcian blue/nuclear fast red for chondrogenesis. Undifferentiated cells were used as a control. hDIAS, human dermis-isolated adult stem. Color images are available online.

each construct, which was removed during medium changes. More nonassembled cells were present with Protocols B and C, resulting in significantly smaller constructs compared with Protocol A; the diameter of constructs derived from Protocols A, B, and C were 2.25 ± 0.28 mm, 1.31 ± 0.10 mm, and 1.05 ± 0.18 mm, respectively.

Constructs derived from all groups exhibited comparable thicknesses; the thicknesses of the constructs derived from Protocols A, B, and C were 0.33 ± 0.05 mm, 0.37 ± 0.09 mm, and 0.40 ± 0.08 mm, respectively (Fig. 4A). Protocol C-derived constructs exhibited insufficient mechanical integrity and handling capacity for mechanical testing, and were therefore excluded from further evaluation. Protocol A- and B-derived constructs were mechanically evaluated (Fig. 4B, C). The permeability of constructs from Protocols A and B was comparable ($30.3 \pm 20.5 \times 10^{-15}$ m^4/Ns and $25.6 \pm 29.0 \times 10^{-15}$ m^4/Ns for permeability, respectively).

Constructs formed by Protocol A exhibited uniform histomorphology with moderate cellularity and homogeneous extracellular basophilic matrix with no evidence of mineralization or ossification (Fig. 5A). Similar morphology with slightly increased cellularity and relatively lower amount of extracellular basophilic matrix was observed in the constructs derived from Protocol B. Constructs derived from both groups stained positively with Alcian blue, with slightly greater staining intensity for Protocol A (Fig. 5A). Immunohistochemical screening for the presence and distribution of type II collagen revealed more uniform cellular and extracellular matrix labeling in the constructs derived from Protocol A, compared with uneven labeling in the constructs derived from Protocol B. Constructs derived from Protocols A and B were also evaluated biochemically (Fig. 5B, C).

Of the examined protocols, Protocol A yielded constructs with the most relevant size for tissue engineering applications, as well as improved biochemical content. In addition, Protocol A yielded more consistent construct biomechanics, as indicated by high standard deviations associated with the mechanical properties of Protocol B-derived constructs. Thus, Protocol A was carried forward to Phase III.

Phase III: the effect of growth factors and substrates on chondrogenic properties of hDIAS constructs

In this phase, the nodule formation pretreatment of Protocol A was examined using non-TCP and ACS compared with a TCP control. In addition, the effects of TGF-β1, IGF-I, and 1% FBS during construct formation compared with TGF-β1 alone were investigated.

Cell and construct properties varied by substrate. Nodules formed on non-TCP and ACS, but cells on TCP grew in monolayer (data not shown). hDIAS constructs cultured in the presence of TGF-β1 showed no significant difference in shape and size compared with those cultured in the presence of TGF-β1+IGF-I+1% FBS (combination treatment) (Fig. 6A). Constructs generated from cells expanded on TCP (TCP-derived constructs) had irregular surfaces, whereas constructs formed from nodules on non-TCP or ACS (non-TCP- or ACS-derived constructs, respectively) were smooth and flat. The diameters and thicknesses of non-TCP-derived constructs, cultured with TGF-β1 or combination treatment, were 1.59 ± 0.07 mm and 0.94 ± 0.10 mm, and 1.66 ± 0.08 mm and 0.88 ± 0.04 mm, respectively. TCP- and ACS-derived constructs exhibited diameter and thickness values of 1.61 ± 0.14 mm and 0.62 ± 0.05 mm, and 1.69 ± 0.11 mm and 0.91 ± 0.03 mm, respectively. TCP-derived constructs were significantly thinner compared with those derived from other substrates.

Metachromatic Toluidine blue staining is indicative of the presence of sulfated GAGs that are the main type of proteoglycans contributing to cartilage compressive strength. Toluidine blue staining was more intense in non-TCP- and ACS-derived constructs with supplementation of the combination treatment (Fig. 6A). Constructs derived from non-TCP and ACS substrates displayed greater staining for type II collagen than TCP-derived constructs.

The gross morphology and histological differences were reflected in the mechanical properties (Fig. 6B, C). The aggregate and shear modulus values of the non-TCP-derived constructs, supplemented with the combination treatment, were significantly higher than those of the TCP-derived or non-TCP-derived constructs supplemented with TGF-β1 only. ACS did not contribute to the mechanical improvement of the constructs over the non-TCP group. Permeability of non-TCP-derived constructs, supplemented with TGF-β1, was $40.85 \pm 29.62 \times 10^{-15}$ m^4/Ns. Non-TCP, TCP, and ACS coatings did not affect the permeability of constructs supplemented with the combination treatment: $26.46 \pm 15.59 \times 10^{-15}$ m^4/Ns, $14.94 \pm 4.92 \times 10^{-15}$ m^4/Ns, and $22.96 \pm 7.75 \times 10^{-15}$ m^4/Ns, respectively.

Discussion

This study aimed to identify strategies to chondroinduce hDIAS cells and to form neocartilage constructs. The multilineage differentiation potential and common stem cell markers of hDIAS cells were identified, and cartilage tissue engineering regimens previously shown to be efficacious in other models were compared and refined.[15–17] The study was performed in three phases. The Phase I hypothesis was that

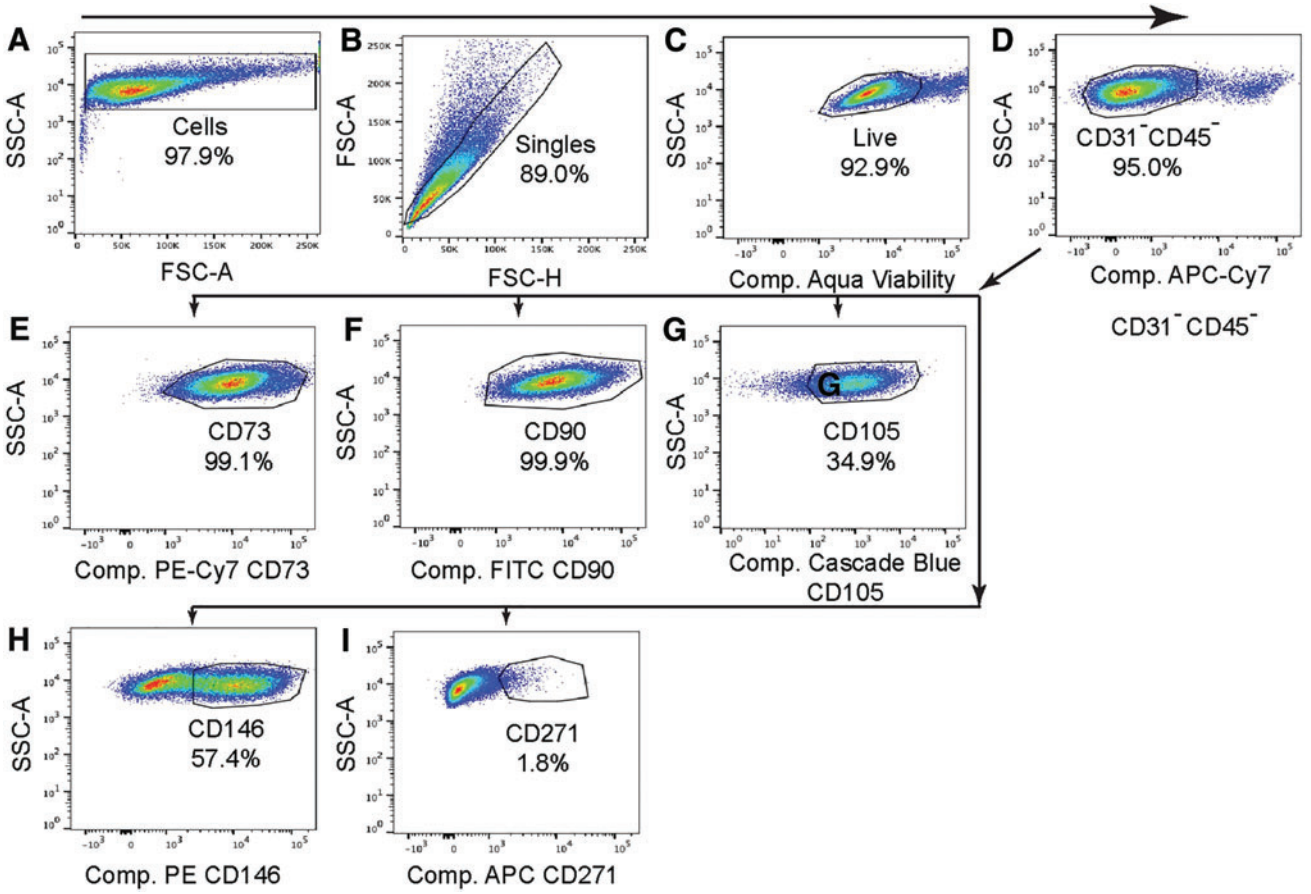

FIG. 3. Flow cytometry assessment for stem cell markers. The following gating strategy was applied, shown by *black arrows*. **(A)** The gate was placed on the general cell population excluding cellular debris by size using forward and side scatter. **(B)** Gate was applied to separate doublets and cell clumps. **(C)** Events staining positive with viability dye were excluded, and gate was placed on aqua blue dim population to select viable cells. **(D)** Within live cells, an additional gate was applied to exclude endothelial cells (CD31) and leukocytes (CD45). Within this gate, the following stem cell markers were assessed: **(E)** CD73, **(F)** CD90, **(G)** CD105, **(H)** CD146, and **(I)** CD271. Comp., compensation. Color images are available online.

abdominal hDIAS cells possess multilineage differentiation capacity and express common MSC markers. hDIAS cells differentiated into osteogenic, adipogenic, and chondrogenic lineages and expressed major MSC markers, CD73, CD90, and CD105, supporting the hypothesis. The Phase II hypothesis was that different chondroinductive pretreatments would influence the ability of hDIAS cells to form constructs. The nodule formation pretreatment yielded constructs with 72% larger diameters compared with DIASphere or Chondrogenic expansion pretreatments and 44% higher GAG content compared with DIASphere pretreatment.

Carrying forward the nodule formation pretreatment, Phase III refined the pretreatment and construct formation steps. The Phase III hypotheses were that refining nodule formation by pretreating hDIAS cells on ACS would enhance their chondrogenic potential and that a growth factor combination efficacious in animal models would be similarly efficacious on hDIAS cells. ACS yielded constructs on-par with non-TCP. Both ACS and non-TCP enhanced construct mechanical properties compared with TCP. In addition, a combination of TGF-β1, IGF-I, and 1% FBS significantly increased the construct aggregate modulus and shear modulus by 75% and 69%, respectively, compared with TGF-β1 alone. The positive response of hDIAS cells to chondroinductive stimuli, as well as the applicability of animal DIAS cell protocols to hDIAS cells, makes hDIAS cells an attractive cell source for cartilage repair and regeneration.

hDIAS cells exhibited stem-like properties, such as trilineage differentiation and expression of MSC markers. These findings are consistent with other stem cells isolated from various dermal niches, reviewed elsewhere.[26] For example, skin-isolated stem cells have been characterized as originating from the hair follicle,[27–29] perivascular,[29] and sebaceous gland niches.[30]

This study's hDIAS cells do not appear to be associated with the hair follicle niche because the abdominal skin-sourced tissue has a low follicle density. Furthermore, the hDIAS cells do not express CD271, a marker for dermal stem cells associated with the hair follicle niche.[27] A high percentage of hDIAS cells expressed CD146, a surface glycoprotein, which, although not considered a classical MSC marker, has been observed on MSCs and pericytes.[31] Therefore, it is plausible for the hDIAS cells to be associated with the perivascular niche due to CD146 expression. These findings support the availability and accessibility of stem cells

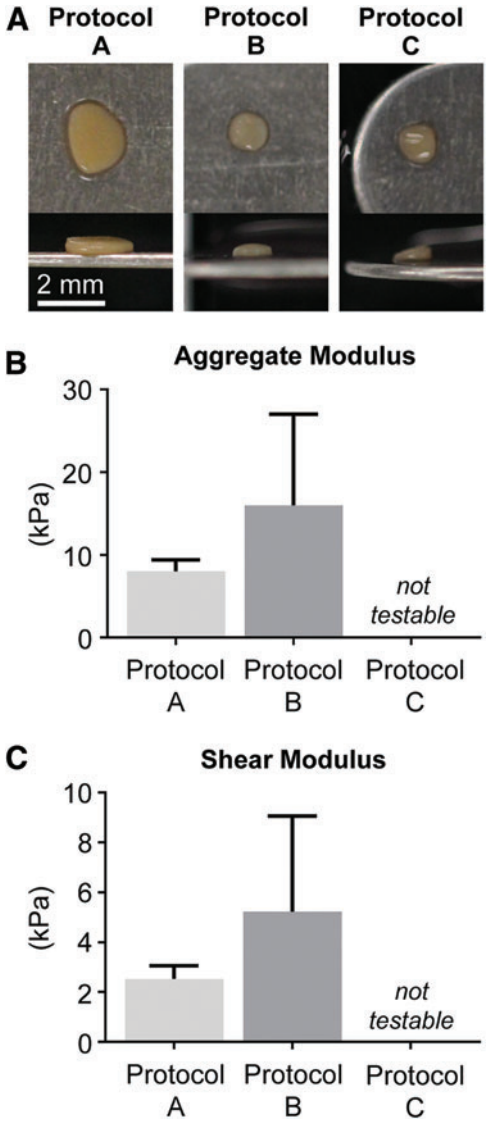

FIG. 4. Gross morphological and mechanical properties of hDIAS constructs generated from cells derived from three pretreatment regimens. **(A)** Gross morphology of top and size views of hDIAS constructs after 4 weeks of culture. **(B)** Aggregate modulus and **(C)** shear modulus of Protocol A- and B-derived constructs after 4 weeks of culture. Protocol C-derived constructs displayed insufficient mechanical integrity and handling capacity for mechanical testing and thus were excluded from the evaluation. Color images are available online.

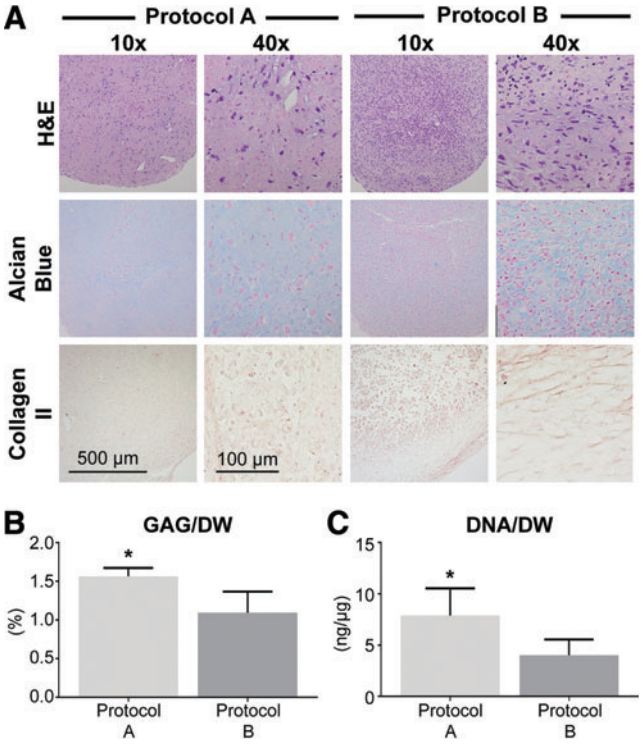

FIG. 5. Histological, immunohistochemical, and biochemical evaluations of hDIAS constructs derived from Protocols A and B. **(A)** H&E, Alcian blue, and type II collagen staining of Protocol A- and B-derived hDIAS constructs after 4 weeks of culture. Biochemical content of hDIAS constructs was also evaluated. **(B)** GAG per DW. **(C)** DNA/DW. The * indicates the protocol that was selected to be carried forward to the Phase III. DW, dry weight; GAG, glycosaminoglycan; H&E, hematoxylin and eosin. Color images are available online.

in adult human dermis, but additional studies are required to pinpoint the niche(s) in which hDIAS cells reside.

hDIAS cells formed nodules on ACS and non-TCP. Previously, it was shown that caprine abdominal skin-derived DIAS cells formed nodules on ACS.[15] Constructs derived from these nodules exhibited abundant cartilage matrix similar to caprine chondrocyte-derived constructs.[15] In contrast, caprine dermal fibroblasts that did not form nodules on ACS did not generate robust constructs. In the present study, hDIAS cells formed nodules, indicating that they respond to ACS (Phase III). hDIAS cells were also able to form nodules of similar size and shape on non-TCP (data not shown). Constructs derived from nodules formed on non-TCP and ACS showed comparable mechanical properties. There may be insufficient receptors to allow hDIAS cells to adhere to non-TCP or ACS, resulting in nodule formation instead of cell spreading. hDIAS cells possess the ability to form nodules and, subsequently, to generate neocartilage constructs.

A 3D culture may be crucial to chondroinduce hDIAS cells for neocartilage formation. Of the three protocols examined in Phase II, only Protocol C did not include culturing cells in 3D before construct formation. Protocol C (chondrogenic cell expansion in monolayer) yielded constructs with insufficient mechanical integrity and handling capacity. Although Protocol B's sphere formation step was intended to enrich the hDIAS cell population,[4] the 3D culture may also have provided a favorable environment for hDIAS cells to retain chondrogenic potential.

Protocol A included nodule formation, which resulted in constructs with superior biochemical and mechanical properties compared with all other protocols. Similarly, in Phase III, hDIAS cells that formed nodules in 3D on non-TCP and ACS yielded constructs with 71% and 46% higher aggregate modulus values, respectively, than chondroinduced hDIAS cells in monolayer on TCP. Mechanical and histological properties of non-TCP- and ACS-derived constructs were comparable; it is plausible that the impact of 3D culture (i.e., the nodule formation) was more dominant than the effect of extracellular matrix substrate in the chondroinduction of hDIAS cells.

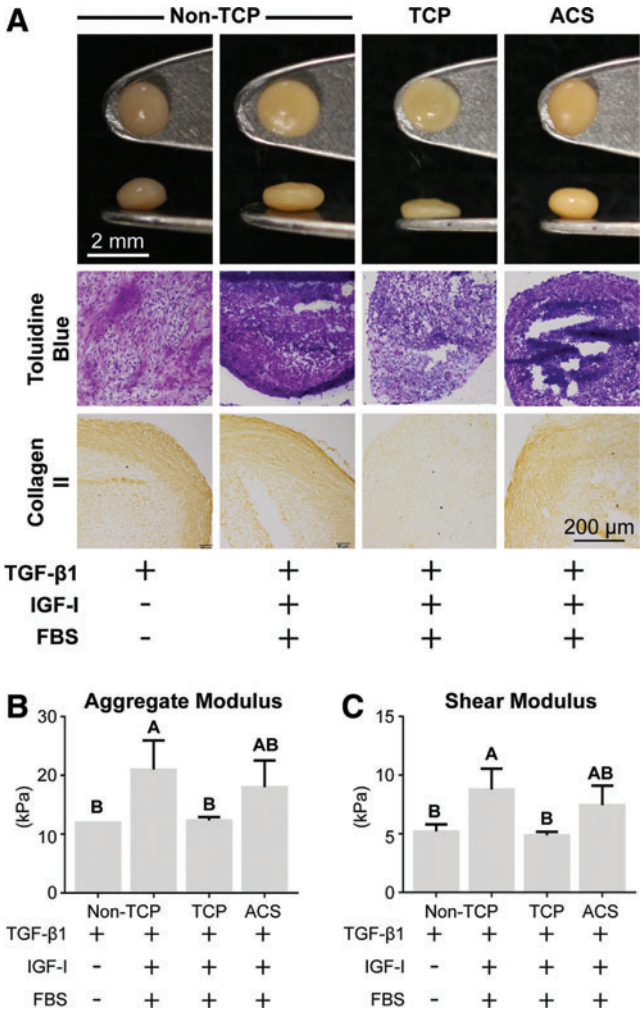

FIG. 6. Gross morphological, histological, immunohistochemical, and mechanical properties of hDIAS constructs generated from cells cultured on different substrates and growth factor supplementations. hDIAS constructs were generated from cells cultured on non-TCP, TCP, and ACS and were maintained in CHG, supplemented with either TGF-β1 only or a combination of TGF-β1, IGF-I, and 1% FBS for 4 weeks. **(A)** Gross morphology of top and size views, Toluidine blue staining, and type II collagen staining of hDIAS constructs after 4 weeks of culture. **(B)** Aggregate modulus and **(C)** shear modulus of hDIAS constructs after 4 weeks of culture. ACS, aggrecan-coated surface; CHG, chondrogenic medium; FBS, fetal bovine serum; TCP, tissue culture-treated plastic. Color images are available online.

In addition, a previous study using foreskin-hDIAS cells showed that Protocol B, followed by an additional 3D chondroinductive treatment, a process called aggregate redifferentiation,[32] yielded constructs with improved aggregate and shear moduli by 2-fold and 1.5-fold, respectively, compared with a control that did not undergo the additional treatment.[17]

While it is not clear how the 3D cultures contributed to chondroinduction of hDIAS cells, the advantages of 3D cultures of MSCs have been shown, for example, to increase survival and multipotency.[33–35] Dedifferentiated chondrocytes in monolayer re-express a chondrogenic phenotype when a spherical cell shape was attained in 3D.[36,37] Modulating cell shape in 3D may also be necessary to effectively chondroinduce hDIAS cells. These findings suggest that 3D environments play a significant role in maintaining chondrogenic potential and effectively chondroinduce hDIAS cells to form neocartilage constructs.

Apart from the pretreatments, growth factors administered during neocartilage formation also greatly influenced the construct mechanical properties. A combination of TGF-β1, IGF-I, and 1% FBS has previously been applied during construct formation, using caprine abdominal skin-derived DIAS cells, in conjunction with the nodule formation pretreatment.[15] Another study using caprine abdominal skin-derived DIAS cells also demonstrated differential effects of TGF-β1, IGF-I, or BMP-2 in construct formation.[16] Specifically, TGF-β1 or BMP-2 treatment significantly increased the GAG content compared with IGF-I, and TGF-β1 treatment significantly increased in the construct compressive properties, up to 14-fold over untreated controls.

In the present study, hDIAS cell nodule formation, followed by an application of TGF-β1, IGF-I, and 1% FBS during construct formation, resulted in 75% and 69% increases in construct aggregate and shear modulus values, respectively, when compared with TGF-β1 alone. Because FBS is known to contain multiple growth factors, including IGFs and TGFs,[38] it remains to be determined which factors in FBS induced the beneficial effects. Nevertheless, these findings suggest that hDIAS cells respond positively to the pretreatment regimens, as well as to chondroinductive growth factors applied during construct formation.

Using hDIAS cells from a single donor is a strength, but also a limitation of this study. Previously, we have published a study comparing the chondrogenic potential of hDIAS cells derived from different anatomical locations, including abdominal skin and different donors.[17] The primary goal of this work was the refinement of chondroinduction regimens and the elucidation of growth factors and substrate effects on functional properties of hDIAS cell-derived neocartilage constructs. Following this, the next logical step would be to see if the optimized protocols reported here are equally efficient across multiple donors.

The GAG content and compressive properties of the engineered constructs in this study are lower than those of native tissue values and require improvement in future studies. In the past, strategies have been demonstrated to improve GAG content and enhance mechanical properties in stem cell-derived neocartilage (e.g., exogenous biochemical factors, hypoxia, shear, and dynamic compression stimuli).[39–41] Due to the structure/function relationship, GAG content and compressive properties typically exhibit a positive correlation,[42] and it is expected that methods to improve GAG content would likewise improve compressive properties. This positive correlation between GAG content and mechanical properties has been demonstrated for both healthy[42] and diseased tissues[43]; for example, as the lamina splendens, or the surface layer of cartilage, breaks down in arthritis,[44] GAG content and compressive stiffness diminish because the damaged collagen network is no longer capable of retaining the GAGs.

It is promising that the methods listed above have not only been useful for improving neocartilage properties but also useful in constructs engineered using stem cells. It would be of value in future studies to examine whether

hDIAS cells would respond similarly to these methods and thus attain properties more similar to native cartilage.

It is of importance not only to improve the functional properties of hDIAS constructs to achieve functional cartilage regeneration but also to validate that hDIAS constructs maintain their phenotypic stability *in vivo*. While hDIAS cells revealed an ability to form neocartilage that exhibits GAG as well as type II collagen *in vitro*, whether these cartilaginous matrices persist after implantation should be evaluated.

Oftentimes, attempts to heal cartilage results in fibrous tissue and fibrous degeneration in the long term; the fibrous fill tissue is characterized by the presence of type I collagen. Thus, although it is not addressed in this study, additional quantitative data on type I collagen would be informative for further optimizations and for future *in vivo* studies. Future studies will be necessary to continue to improve the functional properties of neocartilage derived from hDIAS cells and to elucidate their role in the process of cartilage regeneration in the joint environment *in vivo* before clinical translation.

In conclusion, this study aimed to elucidate the characteristics of hDIAS cells, and to compare and to refine regimens to engineer functional cartilage constructs from hDIAS cells. It was found that hDIAS cells exhibit a multilineage differentiation potential and express stem cell markers. Among the pretreatments explored, Protocol A, which includes a nodule formation pretreatment, was most effective in chondroinducing hDIAS cells to yield constructs with improved construct morphology and chondrogenic properties over other protocols tested. Changing the nodule formation pretreatment step in Protocol A from using non-TCP to using ACS yielded constructs that exhibited comparable functional properties.

Compressive properties of non-TCP- and ACS-derived constructs were increased by 71% and 46%, respectively, compared with those of TCP-derived constructs. In addition, a combined supplementation of TGF-β1, IGF-I, and 1% FBS after the nodule formation pretreatment significantly enhanced aggregate and shear modulus values by 75% and 69%, respectively, when compared with TGF-β1 supplementation alone. Our findings indicate that hDIAS cells possess multilineage differentiation capacity, as well as stem cell markers, and respond positively to chondroinductive regimens to form neocartilage constructs with improved functional properties. Applicability of chondroinductive regimens developed for animal cells to hDIAS cells suggests hDIAS cells be an alternative cell source for cartilage repair and regeneration.

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