

Three-Dimensional Hydrogel Model Using Adipose-Derived Stem Cells for Vocal Fold Augmentation

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Adipose-derived stem cells (ASCs) may provide a clinical option for rebuilding damaged superficial lamina propria of the vocal fold. We investigated the effects of five hydrogels (hyaluronic acid [HA], collagen, fibrin, and cogels of fibrin–collagen and fibrin–HA) on the differentiation of ASCs, with the long-term goal of establishing the conditions necessary for controlling the differentiation of ASC into the functional equivalent of superficial lamina propria fibroblasts. Human ASCs were isolated and characterized by fluorescence-activated cell sorting and real-time polymerase chain reaction. According to fluorescence-activated cell sorting and gene analysis, over 90% of isolated ASCs expressed adult stem cell surface markers and expressed adult stem cell genes. Scaffold-specific gene expression and morphology were assessed by culturing the ASCs in three-dimensional hydrogels. Twofold higher amounts of total DNA were detected in fibrin and cogel cultures than in collagen and HA cultures. Elastin expression was significantly higher in cells grown in fibrin-based gels than in cells grown in other gels. Cells grown in the cogels showed elongated morphology, expressed decorin marker, and exhibited glycosaminoglycan synthesis, which indicate ASC differentiation. Our data suggest that it may be possible to control the differentiation of ASCs using scaffolds appropriate for vocal fold tissue engineering applications. In particular, cogels of HA or collagen with fibrin enhanced proliferation, differentiation, and elastin expression.

Introduction

THE IMPETUS TO IMPROVE vocal fold wound healing and treat vocal fold scar has motivated a number of recent studies where stem cells have been injected into injured vocal folds in animal models. In general, the results have been encouraging with some evidence of improved function and/or improved healing.^{1–3} This is consistent with a large and expanding literature showing that stem cells can aid in tissue regeneration and even reverse fibrosis in diverse organ systems such as lung and liver.^{4,5}

Chemical and physical cues for stem cell differentiation can be provided by substrates used during culture and implantation. Frequently, scaffolds consisting of hydrogels are used for implantation. These scaffolds provide three-dimensional (3D) support and there is evidence that they can stimulate and direct proliferation, differentiation, and eventual regeneration of tissue through chemical and physical cues conveyed to the embedded cells.^{6,7} Therefore, selecting scaffolds that are optimized for specific tissues (e.g., by

mimicking features of the selected tissue) is a critical step in designing tissue-engineered implants.

The design of scaffold materials for implantation of stem cells into the vocal fold has not been explored in detail previously. The Kanemaru group used modified collagen (atellocollagen) as a scaffold,¹ while other groups have injected stem cells without any scaffold.³ Although the use of a scaffold may not be strictly necessary for all types of stem cell therapy, for vocal fold implantation scaffolds can potentially serve double duty: (1) enhancing cell growth and differentiation and (2) restoring volume to the superficial lamina propria with a soft material and thereby immediately improving phonatory function.

With these two goals in mind, collagen and hyaluronic acid (HA) are obvious candidate scaffold materials, since they have been extensively used in tissue engineering, are abundant in the normal vocal fold, and have potential as functional additives to the superficial lamina propria.^{8–11} Collagen and HA scaffolds aid proliferation and differentiation of cells and can be modified or functionalized to

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provide additional physical and chemical cues to guide cell differentiation.^{12,13} Another candidate material is fibrin, a natural biomaterial with important functions during wound healing, including clot formation and recruitment of fibroblasts to wound sites. Fibrin gel has been used as a scaffold for other tissue engineering applications and as a tissue sealant for surgical applications.^{14,15}

Mixtures of scaffold materials or cogels may also offer some advantages. Fibrin–collagen composites have been shown to enhance elongation of vascular smooth muscle cells,¹⁶ for example. By varying cogel composition and proportions, a broader spectrum of gel properties can be explored.

In this study, we assessed the effects of five scaffold materials (collagen, HA, fibrin, cogel of fibrin–collagen, and cogel of fibrin–HA) on proliferation and gene expression of adipose-derived stem cells (ASCs) *in vitro*. The goal of the study was to evaluate whether these gel compositions had significant effects on indicators of ASC differentiation and to provide some preliminary guidance toward optimizing gel parameters for *in vivo* studies.

Our long-term goal is to stimulate differentiation of ASCs toward extracellular matrix–producing vocal fold fibroblasts using a material whose viscoelastic properties can support improved phonatory mucosal function during tissue regeneration.

Materials and Methods

Materials

Blendzyme was obtained from Roche Diagnostics (Indianapolis, IN). Antibodies for fluorescence-activated cell sorting (FACS) analysis were purchased from Invitrogen (Carlsbad, CA; antibodies: CD11b, CD13, CD29, CD31, CD34, CD44, CD45, CD105, HLA-ABC, HLA-DR, and isotypes), BD (Franklin Lakes, NJ; antibodies: CD73, CD90, CD106, and CD166), and (Millipore, Billerica, MA; antibody: ABCG2). Collagen type I gel (bovine, 3 mg/mL) was obtained from BD. Cross-linked HA gel (Restylane, 20 mg/mL) was obtained from Genzyme (Cambridge, MA). Fibrinogen and thrombin were purchased from Sigma (St. Louis, MO). TaqMan Universal polymerase chain reaction (PCR) mix and gene-specific primers were from Applied Biosystems (Foster City, CA). PicoGreen[®] Assay kit and all cell culture products were purchased from Invitrogen.

Isolation of ASCs

Human ASCs were isolated from adipose tissue using collagenase digestion and filtration steps. Human abdominoplasty specimens from 20- to 30-year-old female donors ($n = 5$) were washed five times with saline to remove blood and free fatty acids and then incubated with Blendzyme (0.3 mg/mL) for 30 min while shaking at 37°C. The digested tissue was filtered sequentially through 100 and 40 μ m cell strainers to remove fibrous tissue and then centrifuged at 700 g for 10 min. The cell pellet was resuspended in a lysis buffer (Sigma) to remove red blood cells and then centrifuged for 5 min at 700 g. The cells were resuspended in the culture medium and expanded for 2 weeks to obtain a passage-1 cell population.

Characterization of ASCs

ASCs were characterized by FACS analysis. For FACS, cells were incubated with solutions of fluorescent primary antibodies or their isotypes for 30 min. The excess and non-specifically bound antibodies were removed by multiple washes with phosphate-buffered saline buffer containing 5% serum (v/v). The labeled cells were analyzed using a BD FACSCalibur system.

To test the potential of these cells to differentiate, ASCs were incubated in an adipocyte or osteoblast differentiation medium (Zen-Bio, Research Triangle Park, NC) for 14 days. ASCs grown in control medium (Dulbecco's modified Eagle medium [DMEM], 10% fetal bovine serum, and 1% penicillin/streptomycin) were used as controls. Standard oil red-O and von Kossa stains were used to identify adipocyte- and osteoblast-like cells, respectively.

Transduction of ASCs with green fluorescence protein

To facilitate observation of cells in the 3D culture conditions, the ASCs were transfected with lentivirus-expressing green fluorescence protein (GFP).^{17–19} The GFP lentivirus was produced by transient cotransfection of 293T cells with four plasmids.^{17,20} The ASCs (500,000 cells) were transduced with lentivirus by a single round of infection for 6 h at a multiplicity of infection of 1.0. The efficiency of the lentiviral transduction was confirmed by analyzing GFP expression by FACS (BD). Transfection was repeated three times, with a transfection efficiency of 65–75%. We observed slightly reduced cell proliferation at day 3 in the transfected cells compared with the untransfected cells. However, cell morphology in two-dimensional culture did not appear affected by GFP transduction, which is consistent with a recent report.²¹

Formation of 3D cell–hydrogel constructs

Collagen gel (3 mg/mL) was formed according to the manufacturer's protocol. The collagen solution was neutralized (pH 7.2) with 10 mM NaOH and then incubated in a 37°C incubator to form a gel. The cross-linked HA gel (20 mg/mL) was used as provided (Restylane). Fibrin gel was formed by mixing fibrinogen (100 mg/mL) and thrombin (1 mg/mL). Cogels of fibrin–collagen or fibrin–HA were formed by mixing the components at 1:1 ratios. Passage-3 ASCs (200,000 cells) were mixed with the hydrogels to form cell–hydrogel constructs and were cultured for 7 days. The culture medium was replenished every 2 days.

Real-time PCR

Total RNA was isolated using an RNAeasy system (Qiagen, Valencia, CA) and a bead beater (BioSpec Products, Bartlesville, OK). The RNA was treated with DNase for 20 min to remove genomic DNA contamination from the samples. cDNA was synthesized using oligo-dT primers from the total mRNA (Invitrogen) and 200 ng of the cDNA was used for the real-time PCR analysis (AB7500). TaqMan Universal PCR mix, gene-specific primers (Assays-on-demand[™] products, Applied Biosystems Inc., Foster City, CA), and cDNA were mixed, and the PCR reaction was performed at 50°C for 2 min, 95°C for 10 min, and 40 cycles

of 95°C for 15 s, and 60°C for 1 min. GAPDH was used as an endogenous control. The average cycle threshold (Ct) of three to six replicates was used for the calculation of expression level (relative expression = $2^{-(Ct_{\text{sample}} - Ct_{\text{GAPDH}})}$). Three to six samples from each group were used for gene analysis.

Proliferation assay

Total DNA content was measured using the PicoGreen assay (Molecular Probes, Eugene, OR). The 3D cell-hydrogel construct was suspended in an extraction buffer (1N NH₄OH and 0.2% Triton X-100) and was treated with a bead beater (BioSpec Products) to release DNA. The samples were centrifuged at 10,000 g for 10 min to remove debris, and the extract was used for the PicoGreen assay following the manufacturer's instruction.

Immunohistochemistry

Sections were deparaffinized and antigen was retrieved by heat treatment for 20 min at 95°C in a decloaking chamber (Biocare Medical, Concord, CA). The sections were blocked with 10% horse serum for 40 min at room temperature, and incubated for 1 h at 37°C with 1:25 anti-decorin (Abcam, Cambridge, MA). Antibodies were diluted in phosphate-buffered saline containing 0.5% Tween 20 and 1.5% horse serum. Fluorophore-conjugated secondary antibodies (Texas red-conjugated anti-mouse IgG; Vector Laboratories, Burlingame, CA) were used at 1:200 dilutions.

Glycosaminoglycan assay

Total glycosaminoglycan content was measured using Blyscan Sulfated Glycosaminoglycan assay kit (Biocolor, Carrickfergus, United Kingdom) following the manufacturer's instruction. Note that HA is not detected by this Blyscan assay kit or interfere with the assay.

Image analysis

The 3D cell-gel constructs grown for 7 days were imaged with fluorescence microscopy, and cell shape was measured using ImageJ software (available at <http://rsb.info.nih.gov/ij/>) to assess cell spreading/elongation. Cell fragments that were <91 μm² were not measured. The cells to be measured were fit with ellipses and the ratio of major over minor axis length was used as an indicator of cell elongation.

Statistical analysis

Total DNA and real-time PCR results were analyzed by single-factor analysis of variances to test for overall effects (at a $p < 0.05$ significance level) of scaffold materials (fibrin, collagen, HA, fibrin-collagen, and fibrin-HA), followed by pair-wise comparisons among scaffold materials for total DNA. The real-time PCR results for CD105, CD44, decorin, and elastin were analyzed using Tukey *post hoc* tests with a 95% confidence interval. There was no *a priori* rationale for limiting pair-wise comparisons of particular scaffold materials, so all potential pair-wise comparisons were performed.

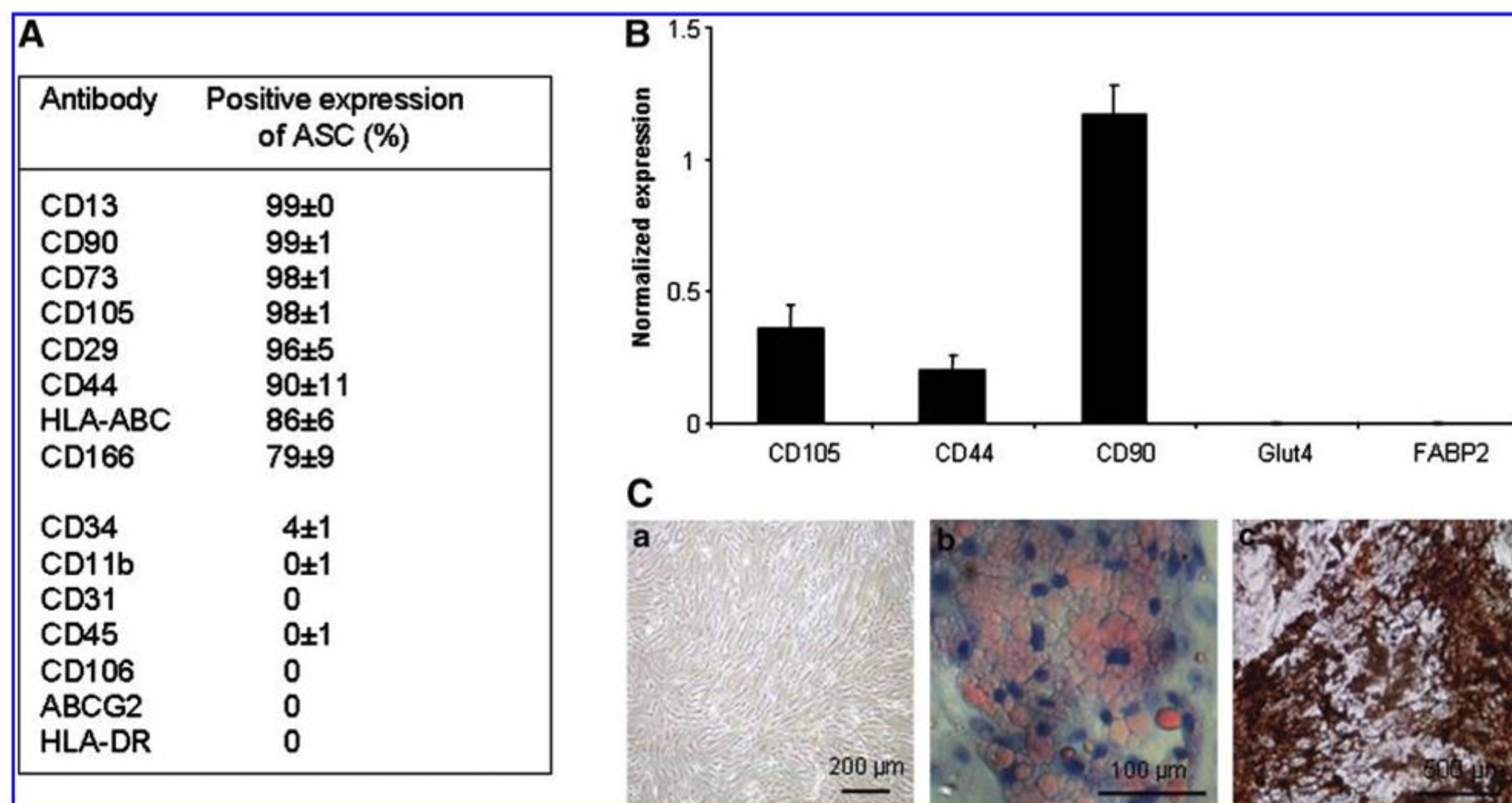


FIG. 1. Characterization of ASCs. **(A)** Expression of surface antigens on ASCs. ASCs (passage 2) were labeled for surface antigens and analyzed by fluorescence-activated cell sorting ($n = 3$). ASCs were hematopoietic-lineage negative but positive for adult stem cell markers. The value represents mean \pm standard deviation. **(B)** Gene expression of ASCs grown in two-dimensional culture. Stem cell genes were expressed in the ASCs, but the expression of adipocyte genes was not detectable. Total RNA of ASCs (passage 2) was used for real-time polymerase chain reaction analysis ($n = 5$). **(C)** Differentiation of ASCs. **(a)** Bright-field image of control ASCs (passage 3). The ASCs were cultured in an adipogenic **(b)** or osteogenic **(c)** medium. The ASCs were positively stained for oil red-O in red **(b)** and for von Kossa in black **(c)**. ASCs, adipose-derived stem cells. Color images available online at www.liebertonline.com/ten.

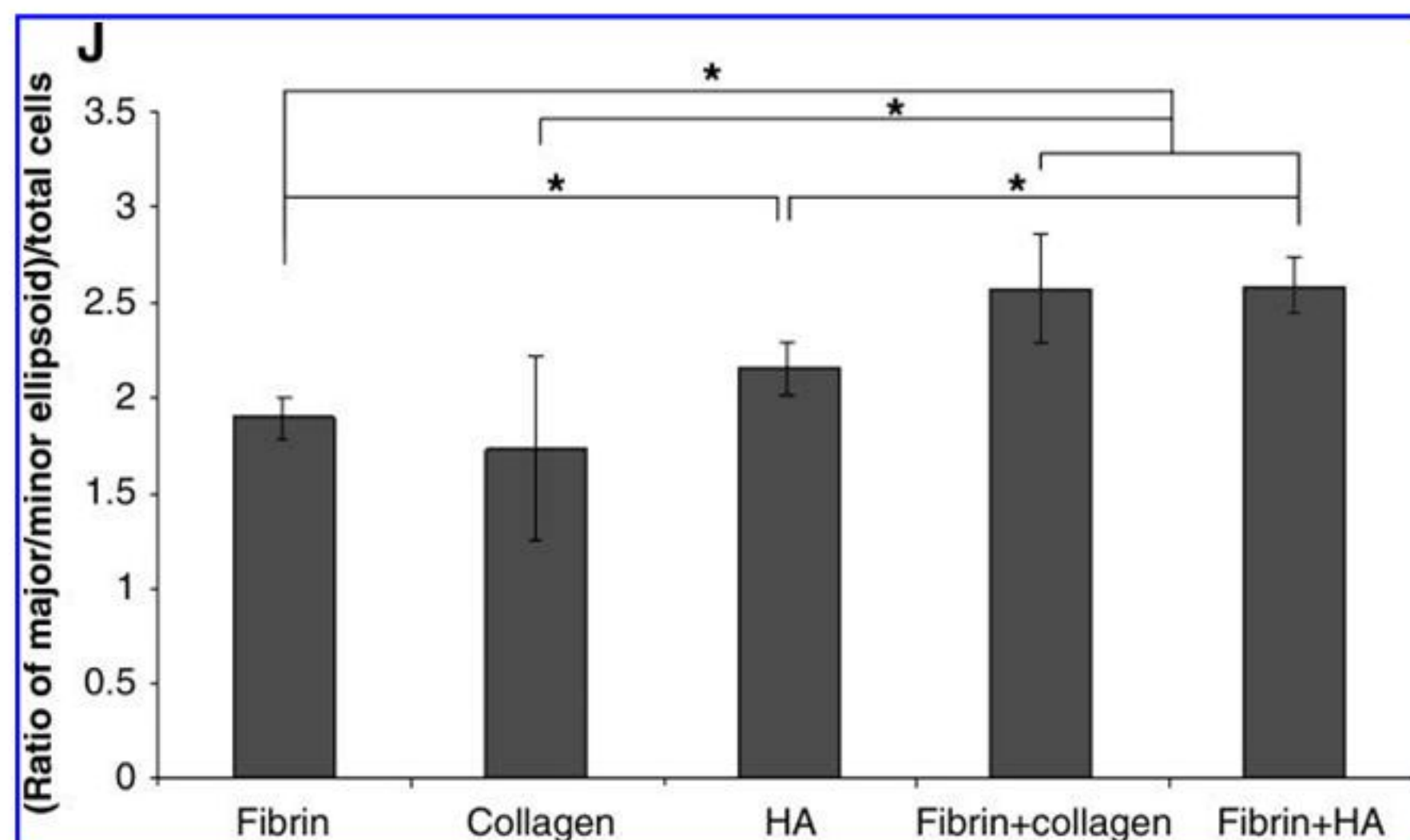
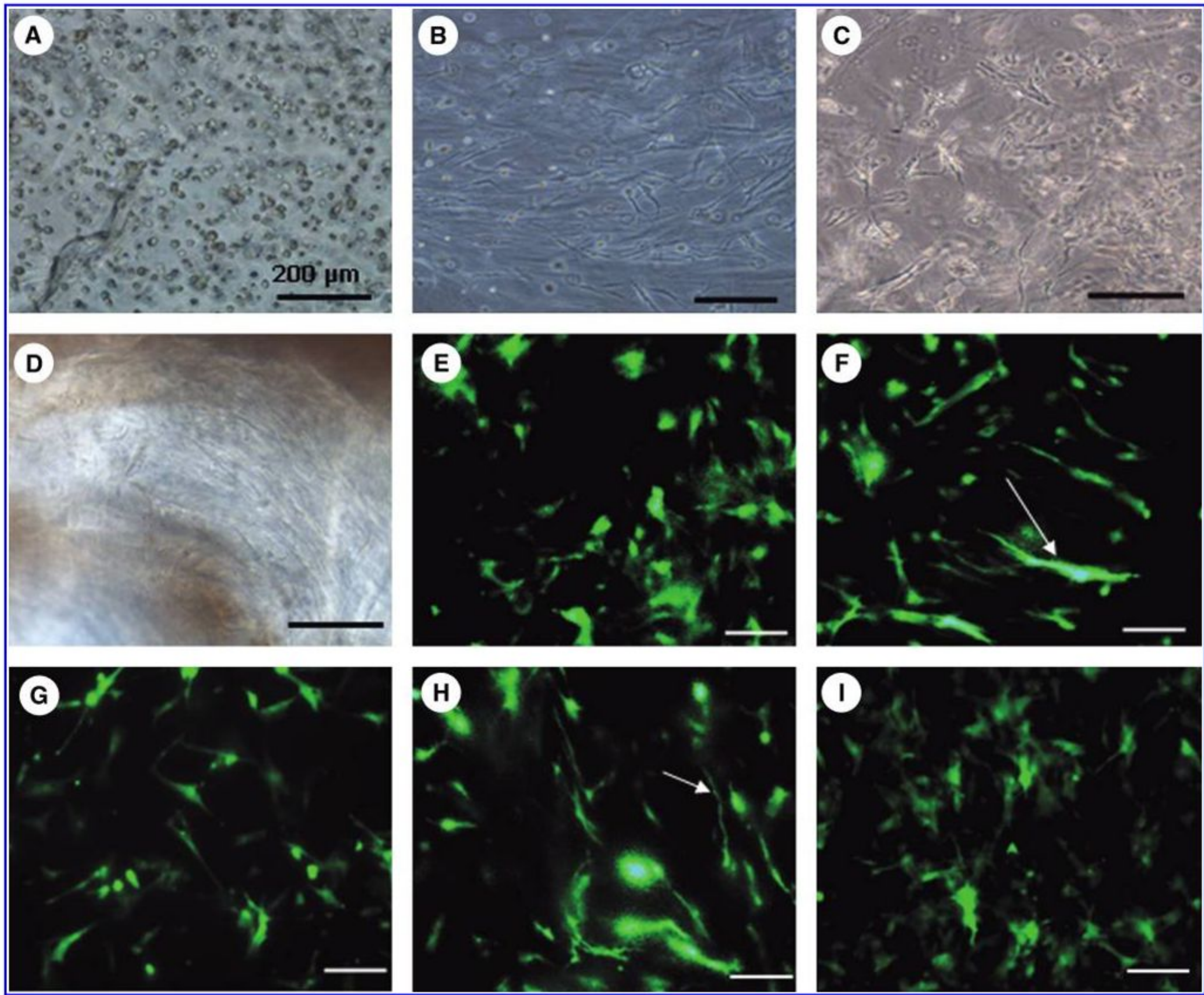


FIG. 2. Cell morphology of green fluorescence protein-transduced ASCs in 3D gels. ASC-green fluorescence protein cells were seeded in fibrin (A, C, E), HA (G), collagen (I), cogel of fibrin-collagen (B, D, F), or cogel of fibrin-HA (H). ASCs were grown for 2 days (A, B) or 7 days (C-I), and images were taken with phase contrast (A-D) or fluorescence microscopy (E-I). Arrows indicate elongated cells. Scale bars, 200 μm . (J) Cell elongation measurement. Five pictures from each group were processed to measure cell elongation using the ImageJ program. Elongation was expressed as the ratio of major over minor length of ellipsoid cells and normalized by total number of cells ($*p < 0.01$, $n = 5$). HA, hyaluronic acid, 3D, three-dimensional. Color images available online at www.liebertonline.com/ten.

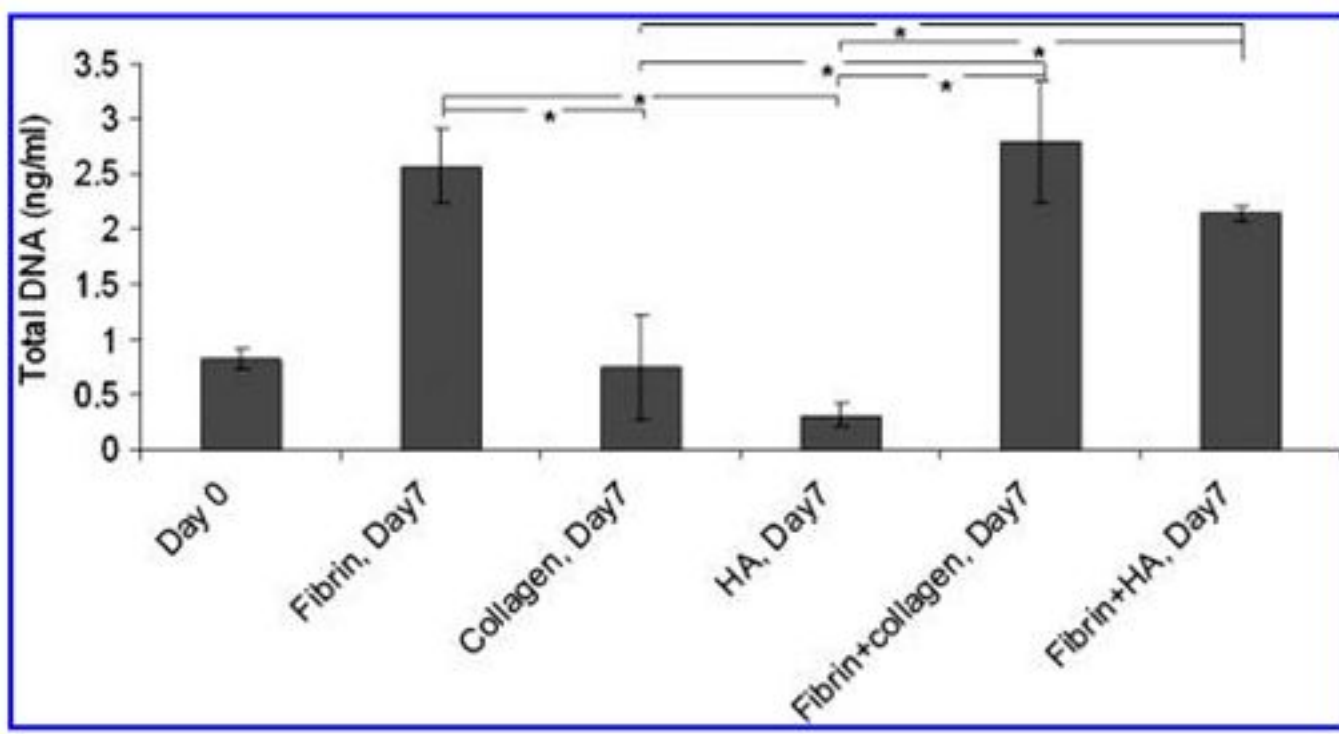


FIG. 3. Proliferation of 3D cultured ASCs. Total DNA isolated from 3D constructs grown for 0 and 7 days was measured using the PicoGreen assay. Samples from the collagen and HA gels had significantly lower DNA content than the samples from the fibrin and cogels ($*p < 0.05$, $n = 3$).

Results

ASC characterization

FACS analysis showed that more than 90% of the isolated cells expressed CD13, CD90, CD73, CD105, CD29, and CD44, that 86% of cells were positive for HLA-ABC, and that 79% of the cells were positive for CD166 (Fig. 1A). Most cells were negative for hematopoietic stem cell markers (CD34 and CD45) and an endothelial cell marker (CD31) (Fig. 1A). This profile of surface marker expression is consistent with a population of cells that contains about 90% ASCs according to published data.^{22–24} To further characterize the isolated cells, expression of stem cell and adipocyte gene markers was analyzed by real-time PCR and immunostaining. Several stem

cell markers (CD105, CD44, and CD90) were highly expressed, but adipocyte markers (Glut4 and FABP2) were barely detectable (Fig. 1B). CD105 showed strong immunostaining, but FABP2 (an adipocyte marker) staining was weak, consistent with the gene analysis (data not shown).

Multipotentiality of the isolated cells was tested by culturing them in an osteogenic or adipogenic medium for 14 days (Fig. 1C). Cells grown in the control medium had a characteristic fibroblast-like appearance (Fig. 1C-a). The cells cultured in the adipogenic medium were positively stained with oil red-O, indicative of adipocytes (Fig. 1C-b), and cells cultured in the osteogenic medium were positively stained with the von Kossa method, indicative of osteoblasts (Fig. 1C-c). The cells grown in the control medium stained negatively for both oil red-O and von Kossa stains. Based on the FACS data, the immunostaining and the tests of differentiation, we conclude that the isolated cells are highly enriched for multipotential cells showing stem cell markers and thus we refer to them as ASCs.

Effect of gel type on embedded cells

Cell shape. The effects of hydrogel materials on the ASCs were first assessed by examining how the elongation of the embedded cells was affected by gel type (Fig. 2). At day 7, cells grown in fibrin (Fig. 2E), HA (Fig. 2G), and collagen (Fig. 2I) gels appeared less elongated than cells grown in cogels of fibrin–collagen (Fig. 2F) and fibrin–HA (Fig. 2H). Automated image analysis of cell shape confirmed greater cell elongation in the fibrin cogels compared with single-component gels of fibrin and collagen ($p < 0.01$; Fig. 2J). No difference in elongation was observed between fibrin and collagen gels.

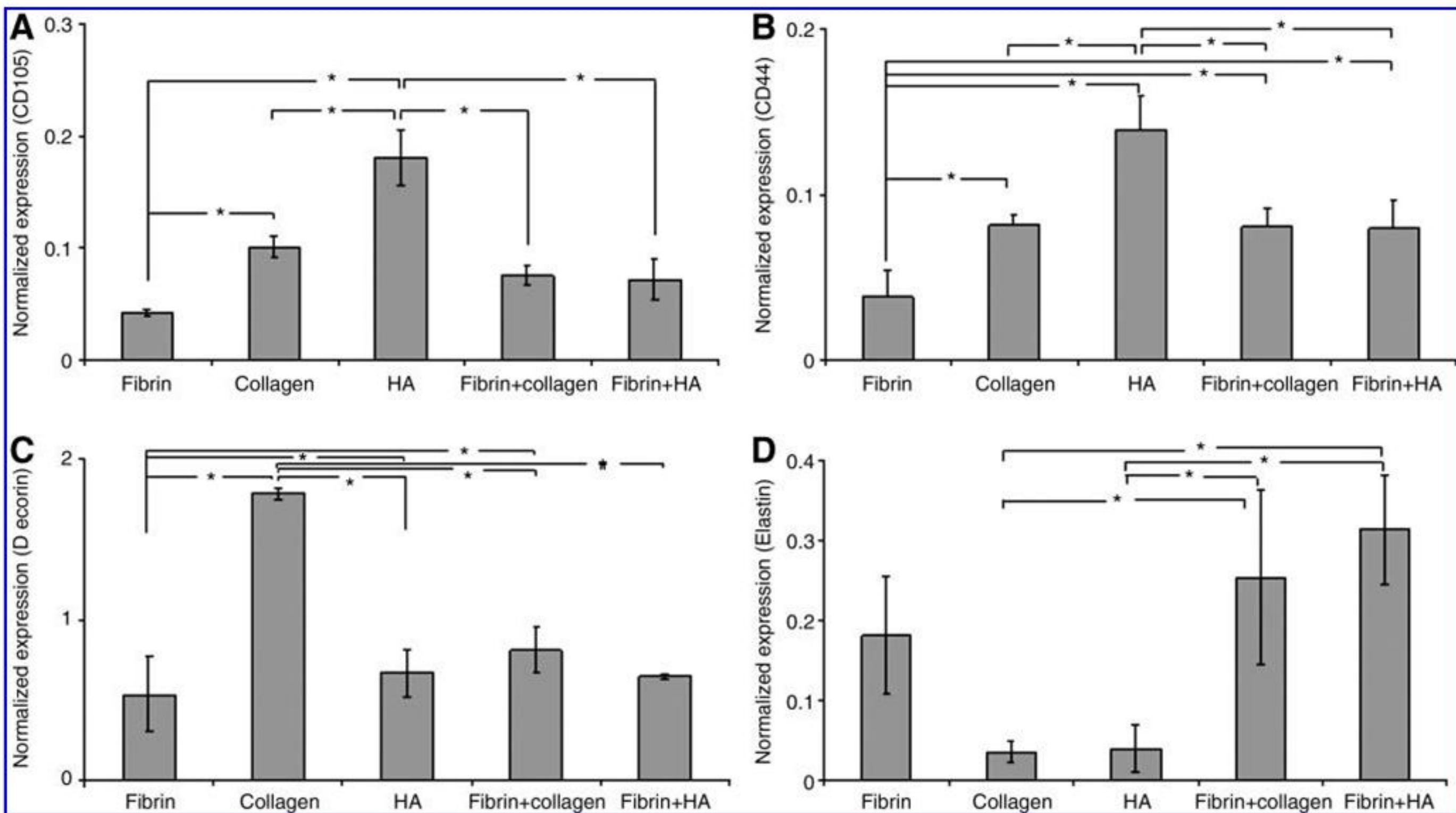


FIG. 4. Gene expression of 3D cultured ASCs. (A) CD105 and (B) CD44 primers were used to detect stem cell gene expression. (C) Decorin and (D) elastin primers were used to detect extracellular matrix gene expression ($*p < 0.05$, $n = 3$).

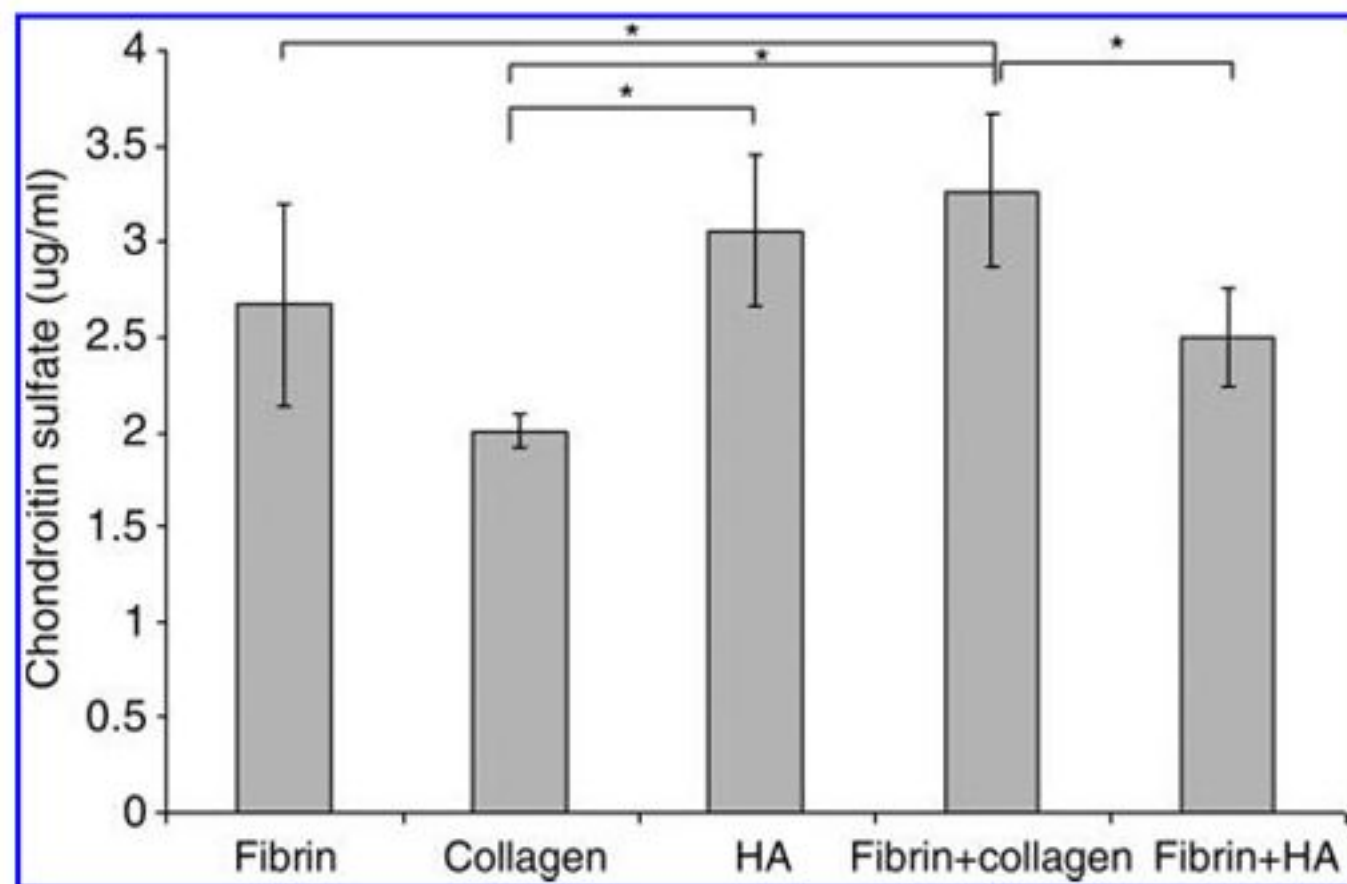


FIG. 5. Biochemical analysis of glycosaminoglycan. Total glycosaminoglycan content from 3D constructs grown for 7 days was measured by dimethylmethylene blue dye binding ($*p < 0.05$).

Proliferation. There was a significant ($p < 0.05$) overall effect of scaffold material on total DNA (Fig. 3). Significant differences among scaffold materials are reported in Figures 3 and 4 for all pair-wise comparisons. The cultures grown in fibrin gel and cogels of fibrin–collagen and fibrin–HA had twofold higher levels of total DNA than the cultures grown in collagen and HA gels, indicating increased cell proliferation (Fig. 3, $p < 0.05$). Cell proliferation was not observed in the collagen and HA gels (Fig. 3).

Surface markers. CD105 and CD44 levels were lowest for cells grown in fibrin gel (Fig. 4A, B, $p < 0.05$). Cells grown

in the fibrin–collagen and fibrin–HA cogels expressed significantly less CD105 and CD44 than cells in the HA gel. Expression of CD105 and CD44 in cells grown in the HA gels was significantly higher than for cells grown in the collagen gels.

Extracellular matrix production. Decorin expression was significantly higher for the cells grown in collagen gel (about twofold on the average) than for cells grown in all other gels (Fig. 4C). In addition, cells grown in the fibrin–collagen gel had higher expression of decorin than cells grown in fibrin alone. Elastin expression by cells in fibrin and cogels of fibrin–collagen and fibrin–HA was significantly higher than by cells grown in collagen and HA gels (Fig. 4D). No significant difference in elastin expression was found among cells grown in fibrin and both cogels. Glycosaminoglycan content was highest for HA gel and fibrin–collagen cogel ($p < 0.05$), and lowest for collagen gel (Fig. 5).

Immunofluorescence staining identified slightly stronger staining for decorin (extracellular matrix marker) in 3D cogel samples than in single-component gel samples (Fig. 6D–F). Most striking was the increased level of extracellular decorin staining in the fibrin–collagen cogel (Fig. 6E).

Discussion

Tissue engineering has brought tremendous opportunities for treatment of tissue injury and disease. Cell-based therapies may be ideal for repair and regeneration of damaged tissues and long-term tissue augmentation. A variety of stem cell types have been derived from embryonic, umbilical cord, bone marrow, and adipose tissues. We have focused on us-

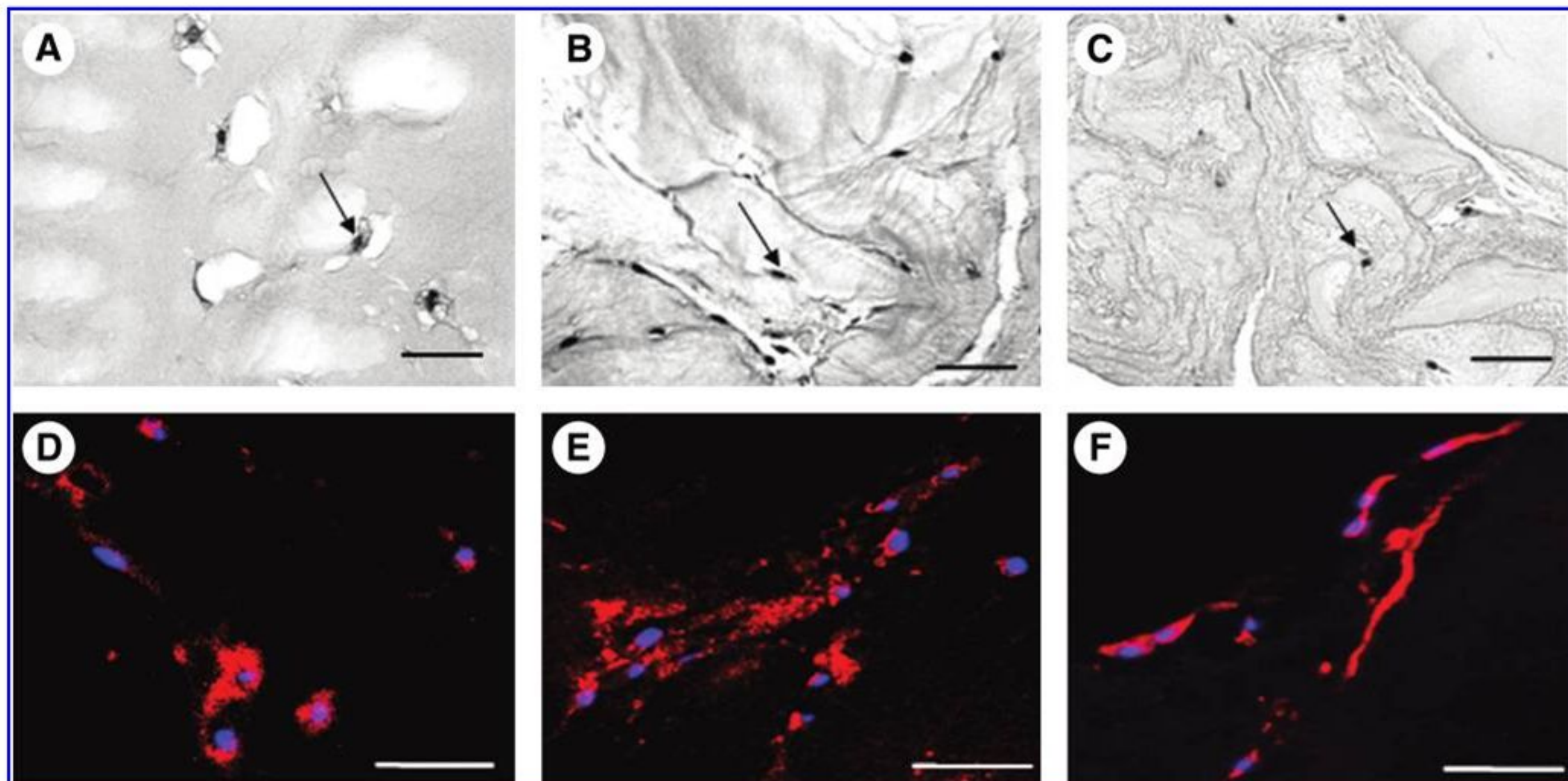


FIG. 6. Immunohistochemistry of 3D cultured ASCs. ASCs were grown in fibrin (A, D), cogel of fibrin–collagen (B, E), or cogel of fibrin–HA (C, F). Top row shows hematoxylin and eosin–stained cells (A–C). Bottom row shows cells stained with anti-decorin (D–F). Decorin-positive cells are red and nuclei are blue. Arrows indicate cells. Scale bars, 100 μ m (A–C) and 50 μ m (D–F). Color images available online at www.liebertonline.com/ten.

ing adipose tissue-derived stem cells because they are abundant and easy to isolate from abdominal subcutaneous fat. They are therefore well suited for autologous transplantation and for differentiation into cell types of mesenchymal origin.²² A recent study by Lee *et al.* showed that autologous ASCs injected into wounded vocal folds improved tissue healing in a canine model.²⁵

It has been argued that an appropriate scaffold is important for stem cell therapy because it improves cell viability and enhances tissue integration.²⁶ Kanemaru *et al.* reported regenerative effects after injecting stem cells mixed with atellocollagen into the canine vocal fold.¹ Several investigators have used stem cells suspended in saline for vocal fold injection.^{2,3,27} To guide our own *in vivo* experiments with stem cells, we tested the five scaffold configurations described here to see if scaffold composition had a significant effect on the encapsulated cells.

Our first set of results demonstrated that the cells we isolated from human abdominal fat could be considered multipotential ASCs. Cell surface antigen analysis using FACS demonstrated that over 90% of the cells were positive for multiple adult mesenchymal stem cell (MSC) markers, consistent with published reports.²² In addition, these cells were negative for CD34, CD45, and ABCG-2 (hematopoietic markers), suggesting that they are not hematopoietic stem cells. Less than 10% of the cells were positively stained for adipocyte marker (FABP2), providing additional evidence that very few adipocytes were present in the isolated cell population. Incubation with the differentiation medium resulted in the differentiation of cells toward the osteogenic and adipogenic lineages. Therefore, we concluded that about 90% of the cells used in tests with the different scaffolds were ASCs, while about 10% were non-ASCs and were likely to be other stromal cells such as fibroblasts.

The ASCs grown in fibrin and collagen gels were observed to be less elongated than the ASCs grown in the cogels of fibrin-collagen and fibrin-HA (Fig. 2), consistent with the data reported by Catelas *et al.*²⁸ Cogels have previously been shown to be favorable for supporting cell proliferation and to achieve mechanical strength not observed in single-component gels.^{29,30} Rowe and Stegemann¹⁶ reported that cogels of collagen and fibrin resulted in higher cell proliferation rates and more extensive cell spreading compared with single-component gels. In addition, the fibrin-collagen cogel significantly increased tensile strength compared with single-component gels.³¹ Our proliferation data support those results: cells grown in fibrin-based hydrogels (fibrin, fibrin-collagen, and fibrin-HA) had higher growth rates compared with cells grown in collagen and HA gels (Fig. 3). This may be due to the stabilization of scaffold materials by increasing compaction and cell-cell contacts within the fibrin gel as Rowe and Stegemann¹⁶ suggested. In addition, we found that HA single gels and cogels of fibrin-HA have lower DNA contents immediately after seeding compared with other gels (Supplemental Fig. S1, available online at www.liebertonline.com). The loss of cells occurs during the initial seeding step and we attribute the loss to less effective encapsulization of the cells in these gel types.

Because a single marker specific to the MSC phenotype does not exist, researchers examine multiple expression of surface markers such as CD13, CD90, CD73, CD105, CD29, and CD44 (but not CD31, CD45, and CD34) as criteria for

MSC identification.³² We have verified such surface marker expression in ASCs used in this article, and further found some of these genes down-regulated when cells differentiated into other lineages (data not shown). We examined expression of CD105 and CD44 to provide some insight into the degree of differentiation resulting from the different culture conditions. CD105 (endoglin) is highly expressed in endothelial cells and MSCs.³³ CD44, an HA receptor, is a cell adhesion molecule mostly expressed in mesenchymal cells and stem cells. Expression of CD44 and CD105 was highest in cells cultured in HA gel. This is consistent with reports that HA supports maintenance of the stem cell state.^{34,35} Therefore, use of an HA scaffold might retard the rate of stem cell differentiation *in vivo*. It was also found that CD105 expression was low for cells grown in fibrin gel, which may reflect a role of fibrin in promoting cell differentiation. These results should be considered as preliminary, because a larger set of markers (e.g., CD13, CD90, CD73, CD105, CD29, CD44, CD31, CD45, and CD34)³² would be needed to better characterize the degree of differentiation of these cells.

Collagen-containing gels were associated with increased decorin gene expression and increased decorin immunostaining, which is not surprising given the known association between decorin and collagen for collagen fibril assembly.^{36,37} Fibrin-containing gels were associated with increased elastin expression. Long and Tranquillo³⁸ found an eightfold increase of elastogenesis in smooth muscle cells when grown in the fibrin gels as compared with collagen gels.

Our results show that five different and relatively simple gel scaffolds produced differential effects on ASCs after 7 days of 3D culture. It appears that the rate of stem cell differentiation might be controllable to some degree by the relative abundance of HA in the scaffold and that fibrin and collagen may be useful components to promote differentiation toward an elongated fibroblast-like morphology. Given the large number of parameters that can be manipulated in creating hydrogel scaffolds, it is clear that further work is necessary to optimize their composition for *in vivo* application. In addition, the viscoelastic properties of scaffolds need to be considered into the selection of materials for vocal fold applications.

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Disclosure Statement

No competing financial interests exist.

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