

# Chondrocytes Cocultured with Stromal Vascular Fraction of Adipose Tissue Present More Intense Chondrogenic Characteristics Than with Adipose Stem Cells

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ORIGINAL ARTICLE

# Chondrocytes Cocultured with Stromal Vascular Fraction of Adipose Tissue Present More Intense Chondrogenic Characteristics Than with Adipose Stem Cells

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Partial replacement of chondrocytes by stem cells has been proposed to improve the performance of autologous chondrocyte implantation (ACI). Our previous studies showed that the increased cartilage production in pellet cocultures of chondrocytes and mesenchymal stem cells (MSCs) is due to a trophic role of the MSCs by stimulating chondrocyte proliferation and matrix production rather than MSCs actively undergoing chondrogenic differentiation. The aim of this study is to compare the trophic effects of stromal vascular fraction cells (SVF) and *in vitro* expanded adipose stem cells (ASC). SVF and culture-expanded ASC ( $n=9$ ) were cocultured with primary human chondrocytes in pellets. By glycosaminoglycan (GAG) and DNA assays, we showed that coculture pellets of SVF and chondrocytes have more GAG deposition than that of ASC and chondrocytes. Results of the short tandem repeats analysis indicated that the increase in the chondrocyte proportion in the coculture pellets is more pronounced in the SVF coculture group than in the ASC coculture group. Using flow cytometry and microarray, we demonstrated that SVF and ASC have different characteristics in cell surface markers and gene expression profiles. SVF is more heterogeneous than ASC, whereas ASC is more enriched in cells from the mesenchymal lineage than SVF. By subcutaneous implantation into nude mice, we showed that constructs of SVF and chondrocytes are better in depositing cartilage matrix than the mixture of ASC and chondrocytes. Taken together, SVF is better than ASC in terms of forming cartilage matrix in pellet coculture and in coimplantation models omitting the need for prior cell expansion. Our study suggests that the SVF in combination with primary human chondrocytes may be a good cell combination for one-stage cartilage repair.

## Introduction

**A**S SELF-REPAIR capacity of cartilage tissue is very limited, full-thickness articular cartilage defects usually lead to the development of osteoarthritis, resulting in serious pain and movement limitations.<sup>1</sup> Current treatments for cartilage defects in young patients include microfracturing,<sup>2</sup> mosaicplasty, and autologous chondrocyte implantation (ACI).<sup>3</sup> However, each of these treatments has limitations.

Fibrocartilage is usually formed after microfracturing, which has inferior mechanical properties compared to native cartilage tissue.<sup>4</sup> Mosaicplasty often causes donor-site morbidity and postoperative pain.<sup>5</sup> The drawbacks of ACI are the necessity of two surgical interventions in a time span of several weeks, which is required for the obligatory cell expansion *in vitro*, dedifferentiation of chondrocytes during this *in vitro* expansion, and unpredictable long-term outcome.<sup>4,6</sup>

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Therefore, clinicians never stopped looking for new therapies that regenerate cartilage defects without the abovementioned issues. With the progress of stem cell biology in recent years, it has been proposed to replace part of the chondrocytes in ACI by other cell sources to avoid the present obligatory, costly, and time-consuming cell expansion phase. Such approach would enable a one-step surgery. Previous studies have mainly focused on bone marrow-derived mesenchymal stem cells (BM-MSCs).<sup>7-9</sup> However, partial replacement of chondrocytes with BM-MSCs is far from optimal because of pain and donor-site morbidity during isolation and the relatively low amount of BM-MSCs in bone marrow aspirates.<sup>10</sup> Other sources of MSCs are, therefore, becoming more and more attractive.

Adipose tissue-derived stromal cells or adipose stem cells (ASC) are attractive sources for cartilage tissue engineering, because they are abundant in adipose tissue, which is more easily accessible than bone marrow.<sup>11</sup> These cells can be easily isolated by liposuction with relatively low morbidity and pain. Most importantly, ASC are, like BM-MSCs, able to differentiate into various cell types of the mesodermal lineage, including adipocytes, osteoblasts, chondrocytes, and myocytes under specific culture conditions *in vitro*.<sup>12</sup> Isolation of ASC usually involves several steps: tissues are first minced, digested with collagenase, and fractionated by differential centrifugation. The resulting pelleted stromal vascular fraction (SVF) is resuspended and then placed in culture flasks<sup>13</sup> to isolate the plastic-adherent ASC subpopulation.

Most researchers have focused on the expanded plastic-adherent ASC subpopulation, while limited work has been done using the minimally processed SVF cell population. Recent reports have indicated that SVF cells and ASC exhibit different features and properties.<sup>14,15</sup> From the perspective of clinical practice, SVF cells have great advantages over ASC, since it is possible to harvest them during the operative procedure itself by processing in the operation theatre and putting them back into the patient without laboratory expansion, which is required for the isolation of ASC. Because adipose tissue is an abundant source of stem cells, cell numbers required for reimplantation can easily be obtained.

In our previous report,<sup>16</sup> pellet coculture of chondrocytes and bone marrow-derived human MSCs (hMSCs) was shown to benefit cartilage matrix formation. In these pellet cocultures, we showed that cartilage matrix genes were mainly expressed by chondrocytes. Furthermore, we showed that the ratio of MSCs decreased dramatically due to massive cell death of MSCs by apoptosis and by stimulation of chondrocyte proliferation in coculture with MSCs. The stimulation of proliferation was at least partly mimicked by culturing chondrocytes in MSC-conditioned medium. These findings were confirmed by an independent study performed by Acharya *et al.*<sup>17</sup>

This, and our study, demonstrated a new mechanism of cellular interaction in coculture pellets of MSCs and chondrocytes. Both studies showed that the beneficial effects on cartilage matrix formation in coculture pellets of MSCs and chondrocytes were due to trophic effects of MSCs stimulating chondrocyte proliferation and cartilage matrix deposition rather than MSCs actively undergoing chondrogenic differentiation. Our follow-up study then showed that these trophic effects are independent of culture conditions and was found in cocultures of chondrocytes with MSCs derived from various tissues.<sup>18</sup> The term trophic effects initially

refer to bioactive molecules produced by nerve terminals, which are not neurotransmitters.<sup>19</sup> In relation to MSCs, the term trophic was first used to describe the process in which MSCs secrete factors that stimulate nearby cells to release functionally bioactive molecules.<sup>20</sup>

Later, the term also relates to the effect of the factors produced by MSC on viability, proliferation, and matrix production of the neighboring cells. This concept has resulted in a paradigm shift in the way MSCs are involved in tissue repair. While traditionally it was believed that MSCs mainly repair damaged tissue by differentiating into specific cell types and replacing lost cells,<sup>21</sup> nowadays the trophic role of the MSC in tissue repair is considered more important.<sup>22</sup> Besides, in cartilage regeneration, these effects were also reported in promoting gain of coordinated functions in stroked brain of rats,<sup>23</sup> stimulating cardiomyocyte proliferation,<sup>24</sup> and vascular regeneration.<sup>25</sup>

Our previous studies demonstrated the trophic effects of expanded ASC.<sup>18</sup> However, the use of ASC in partial replacement of chondrocytes in ACI still needs isolation of adipose tissue by plastic adherence followed by cell expansion before joint surgery. SVF has the advantage that no expansion is required so that it could be easily incorporated into a single-step surgery. In this study, we have compared SVF and ASC as an alternative cell source for replacing part of the chondrocytes in a pellet coculture system and have evaluated cartilage formation *in vivo* in a nude mouse model after subcutaneous implantation. Our data suggested that SVF is a better source than ASC for a coimplantation strategy with primary chondrocytes in cartilage repair. The results of this study shed light on developing a one-step surgical procedure for the regeneration of focal cartilage defects using a mixture of SVF of adipose tissue and uncultured chondrocytes.

## Materials and Methods

### Cell culture and expansion

The use of all human materials in this study has been approved by the Medical Ethics Committee of the VU Medical Center (protocol number 2005/128) after obtaining informed consent. Human chondrocytes were obtained from macroscopically healthy-looking cartilage obtained from knee or hip biopsies of patients with end-stage osteoarthritis undergoing total knee or hip replacement. Cartilage biopsies were digested for 20–22 h in collagenase type II (0.15%) dissolved in chondrocyte proliferation medium. The components of chondrocyte proliferation medium are DMEM (Dulbecco's minimal essential medium) supplemented with 10% FBS, 1× nonessential amino acids, 0.2 mM ascorbic acid 2-phosphate (AsAP), 0.4 mM proline, 100 U/mL penicillin, and 100 µg/mL streptomycin. More details about chondrocyte isolation are described elsewhere.<sup>26</sup>

We defined human primary chondrocytes (CH) in this article as chondrocytes with a low passage number (less than two passages) without immortalization. SVF of human adipose tissue was isolated from the abdominal fat pad of women, who underwent liposuction surgery, according to procedures in previous publications.<sup>27,28</sup> SVF were then seeded in culture flasks with MSC proliferation medium ( $\alpha$ -MEM, minimal essential medium, supplemented with 10% fetal bovine serum, 1% L-glutamine, 0.2 mM ascorbic acid, 100 U/mL penicillin, 10 µg/mL streptomycin, and 1 ng/mL basic fibroblast growth

factor, bFGF). Media were refreshed every 2 days to get rid of nonattached cells. When confluent, cells were trypsinized and passaged. The cells grown on tissue culture plastic were cultured to passage 2 before experimentation.

Expanded cells were called ASC in this article. All reagents used for cell culture were purchased from Gibco, Invitrogen, or unless otherwise stated. Common chemicals were purchased from Sigma-Aldrich.

#### *Pellet culture and chondrogenic differentiation*

For monocultures, 200,000 cells of CH, SVF, or ASC were seeded in one well of a round-bottom 96-well plate (non-tissue culture treated). For cocultures, 200,000 cells were seeded in a ratio of 4:1 (SVF/ASC: CH). Cells were initially seeded in chondrocyte proliferation medium and centrifuged for 5 min at 500 g. Medium was changed to chondrogenic differentiation medium (DMEM supplemented with 40 µg/mL of proline, 50 µg/mL ITS-premix (insulin–transferrin–selenium), 50 µg/mL of AsAP, 100 µg/mL of sodium pyruvate, 10 ng/mL of transforming growth factor beta 3, TGFβ3, 10<sup>-7</sup> M of dexamethasone, 500 ng/mL of bone morphogenetic protein (BMP)6, 100 U penicillin/mL, and 100 µg/mL streptomycin) 1 day after seeding when stable pellets were formed. Cell pellets were cultured for 4 weeks before analysis.

#### *Monoclonal antibody labeling and flow cytometry*

The SVF (after one freeze–thaw cycle) and culture-expanded ASC were analyzed for their cell surface marker expression using monoclonal antibodies (mAb) against human CD29 (cat# 555443), CD31 (cat# 555445), CD34 (cat# 345801), CD45 (Cat# 555483), CD54 (cat# 555511), CD73 (cat# 550257), CD90 (cat# 555595), CD105 (cat# 560839), CD106 (cat# 555647), CD117 (cat# 555714), HLA-ABC (cat# 555552), HLA-DR (cat# 555581), and Lin1 (including CD3, CD14, CD16; CD19; CD20; CD56, cat# 340546) (all from Becton Dickinson Biosciences), CD166 (ALCAM; cat# MCA1926F, AbD SeroTec/MorphoSys), and CD271 (cat# 130-098-103; Miltenyi Biotec BV, Bergisch Gladbach).

All monoclonal antibodies used were of the IgG1 type and either fluorescein isothiocyanate or phycoerythrin conjugated. Cells were washed with phosphate-buffered saline (PBS) and stained with specific antibodies for 30 min at 4°C. Nonspecific fluorescence was determined by incubating cells with conjugated mAb anti-human IgG1 (BD Biosciences). Samples were washed twice and analyzed in a FACSCalibur flow cytometer (BD Biosciences) with the CellQuest Pro software (BD Biosciences).

#### *Histology*

Cell pellets were fixed with 10% formalin for 15 min, dehydrated with ethanol, and embedded in paraffin using routine procedures. A microtome (Shandon) was used to cut 5 µm thick sections. Slides were then deparaffinized and stained for sulfated glycosaminoglycans (GAG) with Alcian blue or Toluidine blue. Nuclei were counterstained with nuclear fast red.

#### *Quantitative GAG and DNA assays*

Cell pellets ( $n=6$ ) were washed with PBS and stored at –80°C for 16–20 h. Subsequently, they were digested in digestion buffer (1 mg/mL proteinase K in Tris/EDTA

buffer (pH 7.6) containing 18.5 µg/mL iodoacetamide and 1 µg/mL pepstatin A) for more than 16 h at 56°C. GAG content was spectrophotometrically determined with 1,9-Dimethyl-Methylene Blue chloride (DMMB) staining in PBE buffer (phosphate buffer with ethylenediaminetetraacetic acid) (14.2 g/L Na<sub>2</sub>HPO<sub>4</sub> and 3.72 g/L Na<sub>2</sub>EDTA, pH 6.5) using an ELISA (enzyme-linked immunosorbent assay) reader (TECAN) at an absorbance of 520 nm with chondroitin sulfate as a standard. Cell numbers were determined by quantification of total DNA using a CyQUANT DNA Kit (Molecular Probes).

#### *Immunohistochemical staining*

For immunohistochemistry, sections were deparaffinized, incubated with 3% hydrogen peroxide, and blocked in 1% bovine serum albumin and 1.5% normal goat serum. Slides were subsequently incubated overnight at 4°C with mouse monoclonal antibodies against COL II (Cat# MAB1330; Novagen). Sequentially, primary antibodies were visualized by EnVision Detection Systems (Dako). Counterstaining was performed with Hematoxylin.

#### *RNA isolation and quantitative PCR*

RNA samples of cell pellets were isolated with the NucleoSpin RNA II Kit (MACHEREY-NAGEL). Total RNA was reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR (qPCR) was performed on cDNA samples using the iQ SYBR Green Supermix (Bio-Rad). PCR was carried out on MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad) under the following conditions: cDNA was denatured for 5 min at 95°C, followed by 45 cycles consisting of 15 s at 95°C, 15 s at 60°C, and 30 s at 72°C. For each reaction a melting curve was generated to test the primer dimer formation and nonspecific priming. The sequences of primers for real-time PCR are listed in Supplementary Table S1 (Supplementary Data are available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)). Calculation of *Relative Expression* was performed with Bio-Rad iQ5 optical system software (version 2.0) using the double delta Ct method.<sup>29</sup> GAPDH primers were used for normalization.

#### *Microarray processing and statistical analysis*

Three donors of SVF were used for the microarray study. Cells from the same three donors were expanded *in vitro* for two passages to obtain ASC. ASC of passage 2 were cryopreserved in liquid nitrogen. The RNA of both SVF and ASC were isolated from the cells immediately after thawing from liquid nitrogen. RNA extraction was performed as previously described. The NuGEN Ovation PicoSL WTA System Kit followed by Encore BiotinIL module were utilized for synthesizing biotinylated sscDNA starting from 50 ng of total RNA. Obtained samples (750 ng) were then hybridized onto Illumina HumanHT-12 v4 Expression BeadChip. Chips were scanned by the Illumina iScan array scanner. Illumina's GenomeStudio v. 2010.3 software was applied to analyze gene expression profiling using the default settings advised by Illumina.

Raw data of fluorescence intensity were normalized by quantile normalization.<sup>30</sup> Differential gene expression was analyzed by the commercial software package GeneSpring, version 11.5.1. (Agilent Technologies). Genes with at least

two-fold difference and being significantly differentially expressed according to a one-way ANOVA with a Benjamini–Hochberg FDR correction and Tukey's HSD *post hoc* test using a cut-off rate of  $p \leq 0.05$  were selected. Changes of gene expression in annotated canonical pathways and biofunctions were analyzed using Ingenuity pathway analysis software (Ingenuity Systems). Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used to predict possible protein–protein interaction (PPI) networks,<sup>31</sup> with a threshold cutoff of a combined score  $>0.4$ .

Clusters were formed using a k-means clustering algorithm. The differentially expressed genes between SVF and ASC with possible PPI were then visualized by constructing a PPI network using Cytoscape software (version 3.2.1, <http://cytoscape.org/>).<sup>32</sup> In the PPI network, each node represented one protein of a differentially expressed gene, the edge represented interaction, and the connectivity degree of a node was defined as the number of interactions between this node and its surrounding nodes.

#### Cell tracking with organic fluorescent dyes

The organic fluorescent dye CM-DiI (Molecular Probes) was used for cell tracking in cocultures. Cells were labeled according to the manufacturer's protocol. Briefly, cells were trypsinized and resuspended in PBS at a concentration of  $2 \times 10^6$  cells/mL. The cells were incubated with 4  $\mu\text{M}$  of CM-DiI at 37°C for 5 min followed by incubation at 4°C for 15 min. Cells were washed with PBS and applied in coculture experiments.

#### 5-ethynyl-2'-deoxyuridine labeling and staining

Cell proliferation in pellets was examined with the Click-iT<sup>®</sup> EdU Imaging Kit (Molecular Probes). Cell pellets were cultured in chondrogenic differentiation medium containing 10  $\mu\text{M}$  EdU (5-ethynyl-2'-deoxyuridine) for 24 h before harvesting. At day 2, cell pellets were washed with PBS and fixed with 10% formalin for 15 min. Sections of 10  $\mu\text{m}$  were cut with a cryotome (Shandon). Sections were permeabilized with 0.5% of Triton X-100 for 20 min and stained for EdU with Alexa-488 cocktail. Nuclei were counterstained with Hoechst 33342 (provided in the same kit).

#### Image acquisition and analysis

Histological images were made with a Nikon E300 microscope. Fluorescent images were taken with a BD pathway 435 confocal microscope (BD Biosciences). Details of image quantification are published elsewhere.<sup>16</sup> Using montage capture, images of high resolutions were obtained covering the entire section of a pellet. Separate images were captured using BP536/40 (Alexa-488), BP593/40 (DiI), and LP435 (Hoechst 33342) and pseudocolored green, red, and blue, respectively. ImageJ software<sup>33</sup> was used for cell counting.

Briefly, we manually set a threshold to avoid artifacts. The number of green cells, red cells, green and red overlapping cells (two colors overlapped), and total cells were counted by running plug-ins written in macro language of ImageJ (available on request). Values represent the mean  $\pm$  standard deviation of at least three biological replicates. To determine the percentage of EDU-positive chondrocytes, SVF or ASC cells in coculture pellets, calculation was carried out as follows: percentage of EDU-positive chondrocytes = overlapped

cells  $\div$  red cell  $\times 100\%$ ; percentage of EdU-positive SFV = (green cells – overlapped cells)  $\div$  (total cells – red cells); percentages of EdU-positive ASC = (green cells – overlapped cells)  $\div$  (total cells – red cells). Since chondrocytes were labeled with red fluorescence, we assumed all nonred cells were SVF or ASC. The actual ratio of red: nonred cells also reflected the input ratio of 1:4 of chondrocyte: SVF/ASC

#### Short tandem repeats analysis

Genomic DNA samples were extracted from pellets with the QIAamp DNA Mini Kit (Qiagen). The sixteen loci of the PowerPlex 16 System (Promega) were amplified, typed, sequenced, and analyzed by ServiceXS BV. Specific alleles for the donor of SVF or ASC and the donor of CHs were identified. These alleles were used to define the origin of cells in allogeneic coculture of SVF/ASC and CH after 4 weeks. The amount of DNA present for each donor was calculated from the areas of the electropherogram for each locus of SVF or ASC and CH-specific alleles and the ratio was determined.

#### Implantation of cells incorporated in alginate gel into nude mice

All animal experiments were approved by the local Animal Care and Use Committee of the University Medical Center, Utrecht (approval no. 104231) and were performed according to the Dutch legislation. Pools of SVF or ASC were made by mixing cells from three donors. CH from another three donors were mixed to make a pool of CH. Then, pools of SVF or ASC were mixed with the pool of CH in a ratio of 4:1, and then resuspended in 2% alginate in PBS at a density of  $1 \times 10^7$  cells/mL. Coimplantation constructs were made by transferring 70  $\mu\text{L}$  of alginate cell suspension to 100  $\mu\text{L}$  of a 100 mM  $\text{CaCl}_2$  solution and jellifying for 5 min at 37°C.

Constructs were washed with PBS and MSC proliferation medium ( $\alpha$ -MEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 0.2 mM ascorbic acid, 100 U/mL penicillin, 10  $\mu\text{g}/\text{mL}$  streptomycin, and 1 ng/mL bFGF). Constructs with only chondrocytes at a density of  $1 \times 10^7$  cells/mL served as positive control, whereas blank constructs without any cells were negative controls. For each condition (SVF+CH, ASC+CH, CH and blank), 10 constructs were made 1 day before the surgery and cultured in chondrocyte proliferation medium (DMEM supplemented with 10% FBS,  $1 \times$  nonessential amino acids, 0.2 mM ascorbic acid 2-phosphate, 0.4 mM proline, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin).

Before the implantation, ten 6-week-old male BALB/C nude mice (Experimental Animal Center, Utrecht) were anesthetized with isoflurane with dosage of 2% for maintenance and up to 5% for induction in oxygen from a precision vaporizer. Then, four subcutaneous pockets were made on the back of a mouse. One construct was put in one pocket. The locations of the constructs were randomized and recorded. At 8 weeks post-implantation, mice were euthanized using  $\text{CO}_2$  in a dedicated chamber, and implanted constructs were then carefully separated from the surrounding fibrous capsule, and washed in PBS for histological analysis and quantitative GAG analysis.

#### Statistical analysis

GAG and DNA quantifications were examined for statistical significance with one-way analysis of variance (ANOVA)

followed by Tukey's HSD (Honest Significant Difference) Test. Statistical analysis of EdU-positive cells was made by using the Student's *t* test. *p* values of <0.05 were considered as statistically significant.

## Results

### *Comparisons of surface marker profiles and chondrogenic potential of SVF and ASC*

We first performed fluorescence activated cell sorting (FACS) to analyze the profiles of CD markers of the SVF and ASC. Representative flow histograms are shown in Figure 1A. The mean percentages of positive cells are displayed in Figure 1B. The SVF fraction contained a subset of cells that were positive for the endothelial cell-associated marker CD31, and the stem cell marker CD34.<sup>34,35</sup> The hematopoietic lineage-associated marker CD45 was weakly present in SVF; however, the CD45-positive cells could not be distinguished as a subpopulation from SVF. As expected, ASC did not express these markers. A fraction of the initial SVF cell population expressed stromal cell-associated markers. Only 10% of the SVF expressed CD105, whereas 86% of the SVF expressed CD90; the levels of CD29, CD166, and CD105 were intermediate to these values.

After isolation of ASC by expansion *in vitro*, the percentage of cells staining positive for each of these markers increased. The initial SVF also contained a subpopulation of cells positive for stem cell-associated markers. An average of 69% of the SVF expressed the stem cell marker CD34. Its level declined to 0.4% in ASC. Expressions of CD73 increased from approximately 50% in SVF to nearly 100% in ASC. In general, ASC contained a more homogenous cell population, which was CD31, CD34, and CD45 negative, but positive for MSC markers like CD105 and CD166. In line with our expectation, SVF represented a heterogeneous cell population containing not only cells with typical MSC characteristics, but also CD31<sup>+</sup> endothelial progenitors, a minority of slightly positive CD45<sup>+</sup> hematopoietic cells and CD34<sup>+</sup> stem cells.

Then, the chondrogenic differentiation potential of SVF and ASC was characterized by putting them in pellet culture. ASC generally formed better aggregates than SVF. We tested nine donors individually. Every donor we tested could give rise to ASC that formed stable pellets, while only six out of nine donors we tested provided SVF that formed stable pellets. Results from histology and GAG quantification (Fig. 1C, D) showed that pellets from ASC produced matrix containing more GAGs than SVF.

### *Chondrocytes cocultured with SVF produce more GAG than with ASC*

To compare the effect of ASC with SVF derived from the same donor on chondrocyte pellet cocultures, both cell fractions were cocultured with primary human chondrocytes at passage 2 (P2) in pellets (SVF+CH and ASC+CH) in a ratio of 4:1. Monocultures of chondrocytes (CH) served as control. Three chondrocyte donors were randomly matched to nine SVF and ASC donor pairs. Results of histology from one representative experiment are shown in Figure 2A. In contrast to pellet monocultures of SVF, in combination with primary human chondrocyte, SVF of all 9 donors formed stable cell pellets. Alcian blue and Toluidine blue staining indicated the presence of GAG in all experimental groups. The staining appeared more intense in the SVF+CH group.

The presence of collagen type 2 was confirmed in all coculture and monoculture groups using immunohistochemistry. CH group had the most intensive staining, while ASC+CH group had the least expression of *COL2A1*. The intensity of *COL2A1* staining in SVF+CH group was intermediate of the other two groups. Overviews of these staining on whole pellets are shown in the insets of Figure 2A. Previously, we showed that GAGs were mainly produced by chondrocytes in coculture pellets.<sup>16,18</sup> When comparing the GAGs between two coculture groups, we ought to eliminate the variant of different donors of chondrocyte, since we focus on donor variation of ASC/SVF. Therefore, instead of using  $\mu\text{g}$  for GAG quantification, relative values of total GAG and GAG/DNA in coculture groups were obtained by dividing absolute values of total GAG or GAG/DNA in coculture groups by to absolute values of the same in chondrocyte (CH) monoculture groups.

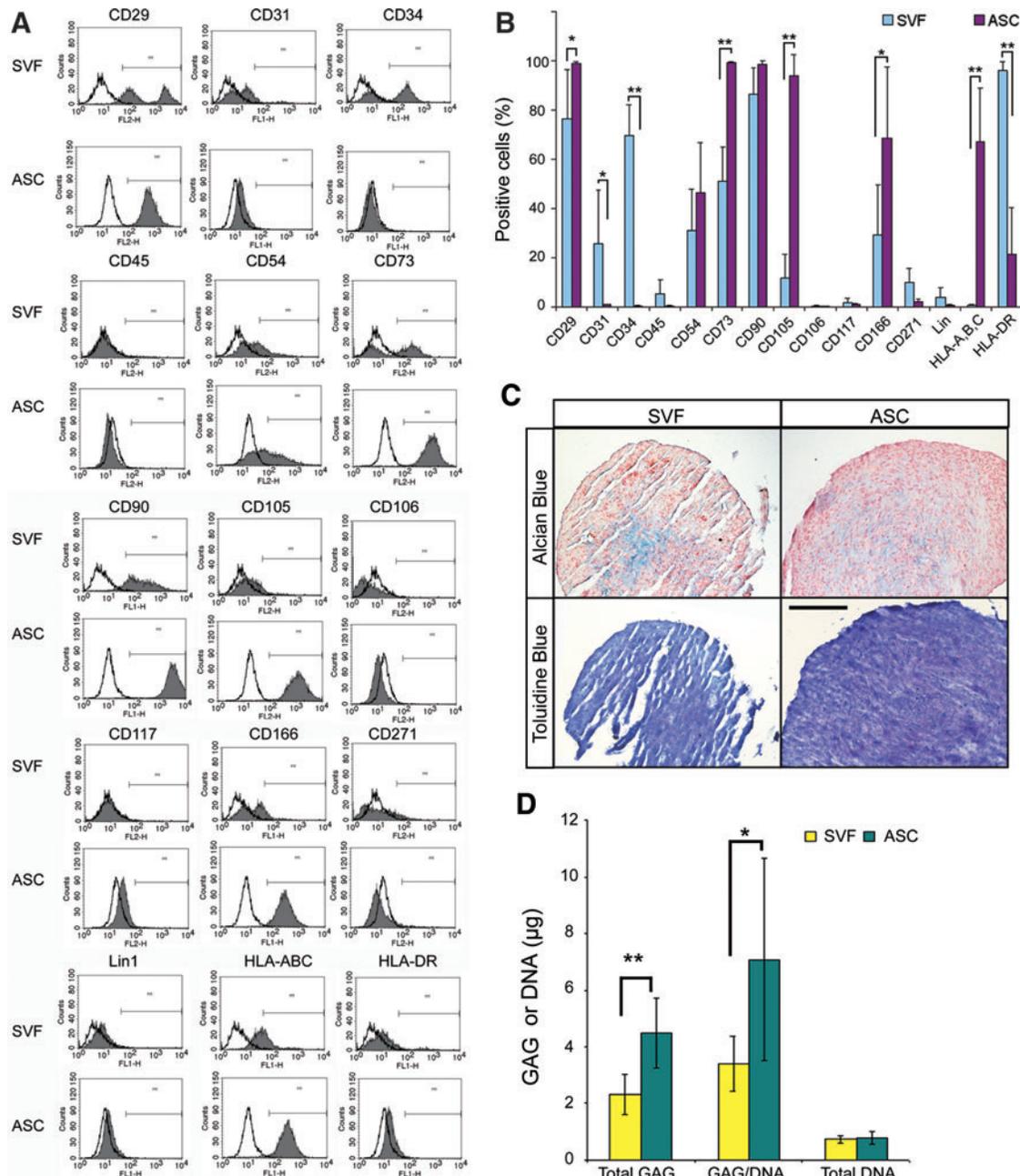
Data from nine SVF or ASC donors are averaged and displayed in Figure 2B. SVF+CH group significantly showed higher capacity in producing GAG than the ASC+CH group. Expression of chondrogenic genes at week 4 of coculture were examined by qPCR (Fig. 2C–E). Expression of *collagen 2a1* (*COL2a1*), *collagen 9a1* (*COL9a1*), and aggrecan (*ACAN*) mRNA tended to be higher in the SVF+CH group, but this did not reach significance.

### *Coculture of chondrocytes with SVF and ASC induces chondrocyte proliferation*

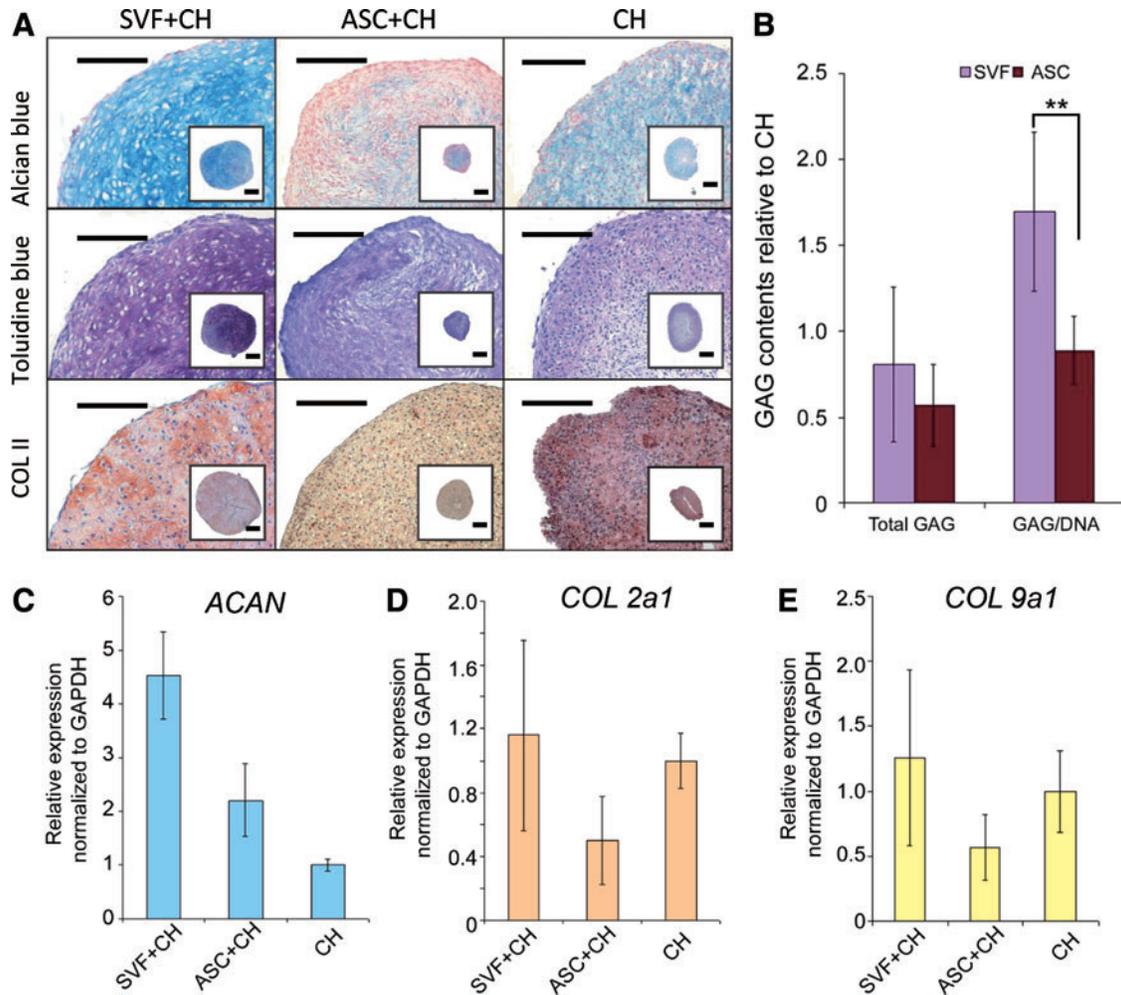
Our previous data showed that proliferation of cells in coculture pellets mainly occurred in the first few days.<sup>16</sup> EdU (5-ethynyl-2'-deoxyuridine) incorporation and cell tracking were used to investigate cell proliferation in the coculture pellets at day 2 after cell seeding. EDU-positive cells were detected in all groups (Fig. 3A left panel). Quantitative data are shown in Figure 3B and C. Percentages of EdU-positive chondrocytes in coculture pellets tended to be higher in coculture pellets with SVF than with ASC, but this did not reach significance most likely due to large inter-donor variation (Fig. 3B). After 2 days of culture, the percentage of EdU-positive SVF or ASC in coculture pellets also did not differ (Fig. 3C).

Previously, we have shown that coculture of primary chondrocytes with bone marrow MSCs induces massive cell death of the latter after 1 week.<sup>16</sup> Therefore, a fluorescent TUNEL assay was performed to determine apoptotic cells in coculture pellets at the end of week 1. High numbers of TUNEL-positive cells were found in cell pellets containing SVF or ASC, but very few in pellet monocultures of chondrocytes (Fig. 3A right panel). TUNEL staining was significantly higher in SVF coculture pellets than in ASC coculture pellets (Fig. 3D). Since cell tracking using organic fluorescent dye did not last for 2 weeks,<sup>16</sup> we measured the overall apoptotic cells per pellet instead of the percentage of apoptotic individual cell types in coculture pellets.

After 4 weeks of coculture, short tandem repeats (STR) analysis was performed to determine the ratio of cells derived from chondrocytes or from either SVF or ASC donors. The distinguishable loci between chondrocytes and SVF/ASC donors were selected and averaged. In SVF coculture pellets, the initial seeding ratio of 4:1 (SVF: chondrocytes) was changed to almost 60% chondrocytes and 40% SVF



**FIG. 1.** Comparison of surface marker profiles and chondrogenic potential between stromal vascular fraction (SVF) and adipose stem cell (ASC). **(A)** FACS was performed to analyze the profile of the expression of CD markers on SVF and ASC. Histograms from one representative donor pair of SVF and ASC are shown. Lines are isotypes, filled graphs indicate specific binding of antibodies. Of each cell fraction at least 100,000 cells were analyzed. **(B)** % of positive cells for each CD marker was calculated based on the average of nine donor pairs of SVF and ASC. Asterisk represents  $p < 0.05$ . Double asterisks represent  $p < 0.01$ . NS = Not Significant. Error bar reflects Standard Deviation (SD). Statistical analysis is done by Student's *t*-test. **(C)** Chondrogenic potential of SVF and ASC is illustrated by histological staining. 200,000 cells of SVF or ASC were seeded per well in a round bottom nonadherent 96-well plate. Cell pellets were made by centrifuge at 500 *g* for 3 min. Then pellets were cultured in chondrogenic differentiation medium for 4 weeks. Scale bar = 100 µm. Results of a representative donor pair of which SVF formed a stable pellet is shown. **(D)** At 4 weeks of chondrogenic differentiation glycosaminoglycan (GAG) and DNA in the cell pellets were quantified. Data represent the average of five donor pairs of SVF, which formed stable pellets, and ASC. Asterisk represents  $p < 0.05$ . Double asterisks represent  $p < 0.01$ . Error bar reflects Standard Deviation (SD). Statistical analysis is done by Student's *t*-test. Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)



**FIG. 2.** Coculture of chondrocytes with SVF or ASC-enhanced cartilage matrix formation. **(A)** Alcian blue and Toluidine blue staining indicated the presence of GAGs. Immunohistochemical staining showed expression of collagen type II. Cell pellets were cultured in chondrogenic differentiation medium (as described in Materials and Methods section) for 4 weeks before examination. Scale Bar = 100  $\mu$ m. Results of a representative donor pair is shown. **(B)** GAG quantification showed that the SVF+CH group produced more GAG than the ASC+CH group. Data represent the average of nine donor pairs of SVF (or ASC) with chondrocytes. Values are relative to pellet monoculture of chondrocytes (CH).  $p$  value was calculated by Student's  $t$ -test. Error bar reflects SD. Double asterisks represents  $p < 0.01$ . **(C–E)** Expression levels of *ACAN* (C), *COL2a1* (D), and *COL9a1* (E) mRNA were examined by qPCR. RNA samples were extracted from nine donor pairs of SVF and ASC with chondrocytes, from pellets cultured in chondrocyte proliferation medium for 4 weeks. RNA was isolated from three pellet cultures per condition. Relative expression levels were obtained by normalization to GAPDH. Values represent the relative gene expression compared to monoculture of chondrocytes (CH). Error bar reflects SD. Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

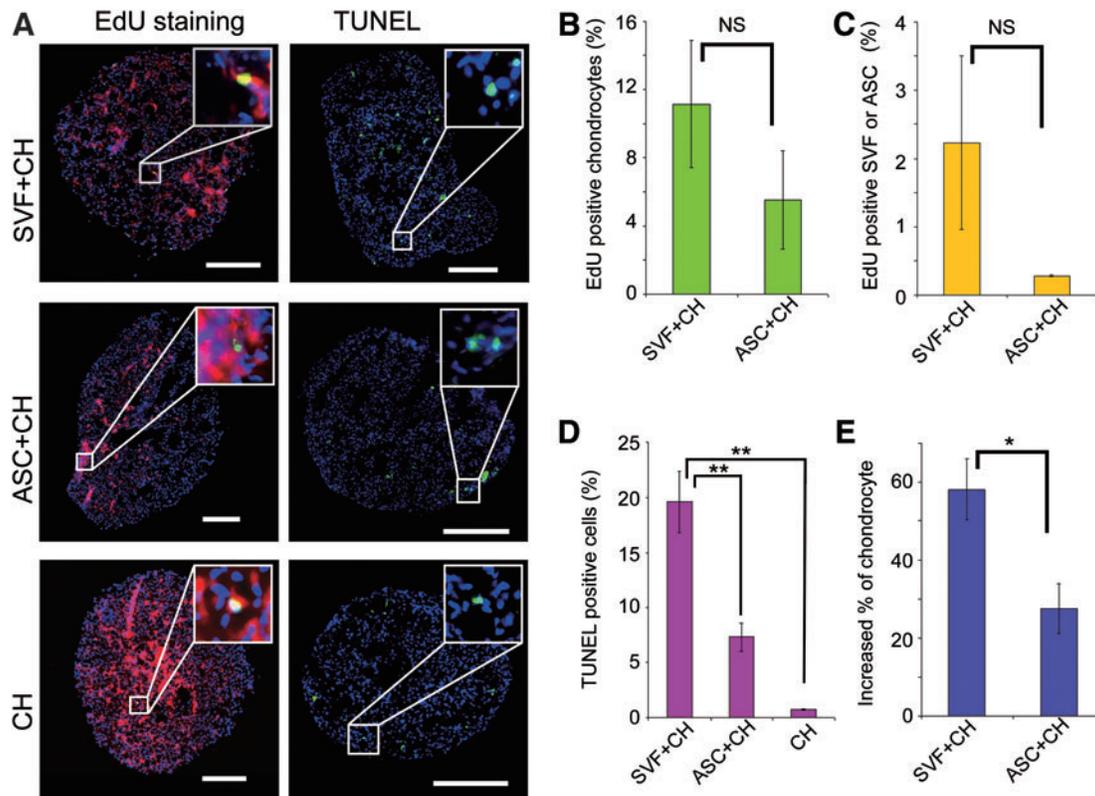
after 4 weeks of culture. The seeding ratio in the ASC coculture pellets after 4 weeks of culture (75% ASC and 25% chondrocytes) did not significantly deviate from the initial seeding ratio of 4:1 (ASC: chondrocytes) (Fig. 3E). Increased cell death of SVF cell fractions in combination with a larger stimulatory effect on chondrocyte proliferation are most likely responsible for the relative overgrowth of the primary chondrocytes in the coculture pellets with SVF.

#### Comparison of global gene expression profiles between SVF and ASC

We have shown that trophic factors of bone marrow-derived MSCs are responsible for increased chondrocyte

proliferation and matrix deposition in coculture pellets with chondrocytes.<sup>16</sup> We, therefore, compared the global gene expression profile of pairs of SVF and ASC to identify differentially expressed genes between both cell populations using a microarray experiment. As shown in Figure 4A, the majority of genes that can be detected in either cell type fall in the region of two-fold change (up or down, yellow dots). Eighty genes were found >two-fold upregulated (red dots) in SVF, whereas 48 genes were >two-fold downregulated (blue dots). Differential expression of a random selection of genes was validated using qPCR (Fig. 4B, C).

Some variation in absolute fold change as determined by microarray or qPCR was observed, however, the trends were the same. A complete list is given in Supplementary



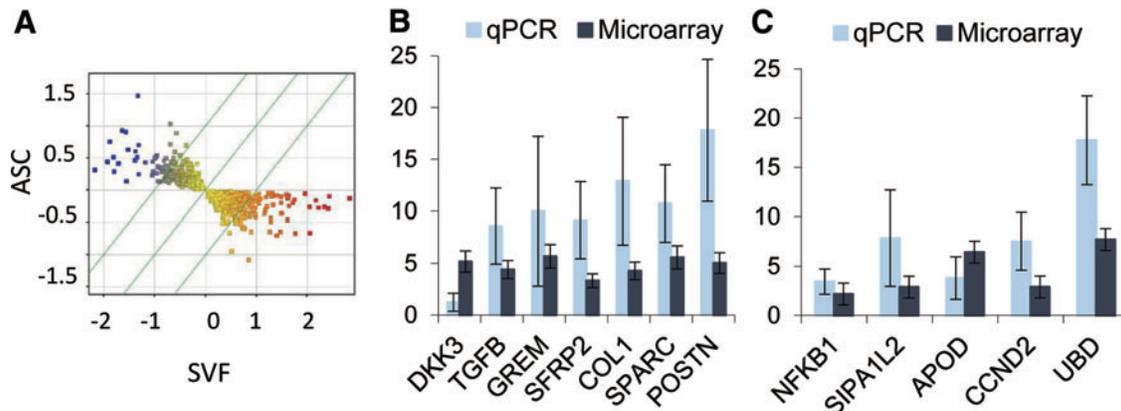
**FIG. 3.** Coculture induces apoptosis of MSCs and proliferation of chondrocytes. (A) *Left panel:* EdU staining of pellets at day 2 after seeding. PCs were labeled with CM-DiI (red). EdU incorporation into newly synthesized DNA was visualized by Alexa-488 (green). Nuclei were counterstained with Hoechst 33342 (blue). *Right panel:* TUNEL staining of pellets were performed at 1 week after seeding. TUNEL-positive cells were visualized with fluorescent labeling (green). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar = 100  $\mu$ m. A representative picture of 1 ASC or SVF donor is shown. *Inserts* showed the *boxes* in higher magnification. (B) Quantification of EdU-positive chondrocytes. Three pellets for each donor pair of SVF or ASC and chondrocytes were analyzed. Data represent the average of three donor pairs. Values expressed relative to monoculture of chondrocytes. *Asterisk* represents  $p < 0.05$ . *Double asterisks* represent  $p < 0.01$ . NS = Not Significant. Error bar reflects SD. (C) Quantification of EdU-positive SVF or ASC. Three pellets for each donor pair of SVF or ASC and chondrocytes were analyzed. Data represent the average of 3 donor pairs. *Asterisk* represents  $p < 0.05$ . *Double asterisks* represent  $p < 0.01$ . NS = Not Significant. Error bar reflects SD. (D) Quantification of TUNEL-positive cells. Three pellets for each donor pair of SVF or ASC and chondrocytes were analyzed. Data represent the average of three donor pairs. *Asterisk* represents  $p < 0.05$ . *Double asterisks* represent  $p < 0.01$ . NS = Not Significant. Error bar reflects SD. (E) STR analysis was performed on genomic DNA isolated from coculture pellets of SVF or ASC and chondrocytes at an initial seeding ratio of 80:20 after 4 weeks of culture. Only informative loci that could discriminate between the SVF or ASC donor and PCs were taken into account. Averages of these loci were calculated to indicate the ratio of SVF or ASC and chondrocytes after coculture. Data represent an average of three donor pairs. *Asterisk* represents  $p < 0.05$ . Error bar reflects SD. Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

Table S2 showing all upregulated and downregulated genes with a more than two-fold change and a  $p$ -value  $< 0.05$  as determined by a one-way ANOVA corrected for a Benjamini–Hochberg FDR and Tukey’s HSD *post hoc* test. As a result of the heterogeneous populations, genes higher expressed in SVF than in ASC are a combination of markers found in endothelial cells (e.g., *EFNB2*), adipocytes (e.g., *APOD*, *FABP4*), and lymphocytes (e.g., *LTA*, *LITAF*).

On the other hand, ASC showed higher expression of genes of the mesenchymal cell lineages when compared to SVF. These genes included *DKK3*, *TGFBI*, *GREM1*, and *SFRP2* that are expressed in chondrocytes,<sup>36–38</sup> and *COL1A1*, *SPARC*, and *POSTN* that are expressed in osteoblasts.<sup>39,40</sup> Roughly, one third of these differentially expressed genes are secreted factors, as shown in Table 1.

Some of the genes together with others (*NFKB1* and *UBD*) on the top of the differentially expressed gene list were selected for qPCR validation (Fig. 4B, C). Variations between the data obtained from microarray and qPCR were observed, however, the trends were the same.

The list of all upregulated and downregulated genes with more than two-fold change was then imported into ingenuity pathway analysis software to identify differential expression between SVF and ASC in annotated canonical pathways and biofunctions. Significantly changed pathways were diseases and disorders (Supplementary Fig. S1), molecular and cellular functions (Supplementary Fig. S2), and physiological system development/function (Supplementary Fig. S3). In addition, the differentially expressed genes were used as input into Search Tool for the Retrieval of Interacting



**FIG. 4.** Comparison of global gene expression profiles of SVF and ASC. (A) RNA samples were isolated from SVF or ASC of three donor pairs and applied in a microarray study. The average ratio of gene expression in SVF over ASC is plotted. *Green lines* indicate thresholds for up (>two-fold) and downregulated gene expression (<two-fold). In *red* are genes depicted which are >two-fold upregulated in SVF. In *blue* are genes depicted which are >two-fold downregulated in SVF. (B) Expression levels of genes that were upregulated in SVF were validated by qPCR. The values are expressed relative to ASC and represent the mean of three donors. (C) Expression levels of genes that are upregulated in ASC were validated by qPCR. The values were relative to SVF and represent the mean of three donors. Error bar reflects SD. Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

Genes/Proteins to identify predicted interaction networks of genes and/or proteins (Supplementary Fig. S4).

The results show three major clusters of interacting proteins. The first one (red) is a group of secreted factors centered by *FNI* and *TGFBI*, most of which are produced by cells from the mesenchymal lineage to regulate the differentiation and proliferation of chondrocytes. One cluster (brown) of extracellular matrix (ECM) components centered on *COLIA2* is also found. The third major cluster (yellow) is a group of transcription factors and cytokines, which is regulated by *NFKB1*.

#### Ectopic cartilage formation of SVF and ASC mixed with chondrocytes

To evaluate our previous findings *in vivo*, mixtures of chondrocytes and SVF or ASC (ratio 1:4) were incorporated in an alginate gel and subcutaneously implanted in immunotolerant mice.<sup>41</sup> Eight weeks after implantation, there was a significant accumulation of ECM components in the pericellular area in all experimental groups. SVF+CH group produced most ECM in comparison with the other two groups according to the color of the staining (Fig. 5A left and middle panels).

Immunohistochemical staining indicated the presence of more collagen type 2 protein expression in engineered cartilage of SVF+CH than in ASC+CH (Fig. 5A right panel). Results of GAG assay confirmed that SVF+CH group contained more GAG than the other two groups (Fig. 5B). The quantification of DNA in the constructs showed that SVF+CH had fewer cells (Fig. 5C). These data suggested that the SVF mixed with chondrocyte produced more cartilage matrix than ASC *in vivo*.

#### Discussion

In previous reports, we have shown that coculture of MSCs and CH in pellets augmented cartilage matrix for-

mation. This effect can be largely attributed to a trophic effect of the MSCs that increases the proliferation and matrix formation by chondrocytes, rather than by chondrogenic differentiation of MSCs. We furthermore showed that this trophic effect of MSCs is a general feature that can be observed in MSCs from multiple sources like bone marrow, adipose tissue, and synovium.<sup>18</sup>

In this study, we investigated for the first time the trophic effects of SVF of adipose tissue. We show that the SVF is a better cell source than ASC in facilitating cartilage formation in coculture with chondrocytes. This conclusion is based on the following observations; (1) Coculture pellets of SVF and chondrocytes produced more GAG than pellets of ASC and chondrocytes, (2) the proportion of chondrocytes after coculturing is more increased in coculture pellets of SVF and chondrocytes than pellets of ASC and chondrocytes, (3) mixtures of SVF and chondrocytes deposit more GAG and collagen type 2 than that of ASC and chondrocytes in an ectopic cartilage formation model in nude mice.

For the first time, we showed in this study that SVF cells could act as trophic mediators in coculture pellets with chondrocytes. We previously reported that expanded cells isolated from SVF (adipose-derived MSCs) could induce chondrocyte proliferation in coculture pellets and promoted GAG formation in cocultures.<sup>18</sup> In this study, we showed that in comparison to ASC, SVF from the same donor were more potent in promoting GAG formation in cocultures. The proliferation rate of chondrocytes tended to be higher in coculture with SVF, but this did not reach significance. This is likely explained by considerable inter-donor variation. However, we observed another interesting phenomenon: more cell death was found in SVF+CH than in ASC+CH coculture pellets.

This could possibly explain why on an average GAG production of each cell (GAG/DNA) is higher in SVF+CH than in ASC+CH. Increased cell death of SVF, possibly in

TABLE 1. DIFFERENTIALLY EXPRESSED SECRETED FACTORS WITH MORE THAN 2 FOLDS CHANGE

<i>Symbol</i>	<i>Accession</i>	<i>Fold change (ASC vs SVF)</i>
APOD	NM_001647.2	-6.43455
PLA2G7	NM_005084.2	-5.71599
EFNB2	NM_004093.2	-4.92421
CXCL5	NM_002994.3	-4.32704
FABP4	NM_001442.1	-3.32248
LTB	NM_002341.1	-2.79883
CFD	NM_001928.2	-2.55915
THBD	NM_000361.2	-2.55425
IL32	NM_001012636.1	-2.48646
LITAF	NM_004862.2	-2.37264
FABP3	NM_004102.3	-2.2869
NID1	NM_002508.2	-2.19089
APOC1	NM_001645.3	-2.15423
LTA	NM_000595.2	-2.14469
ATF3	NM_001040619.1	-2.13038
IGFBP7	NM_001553.1	2.019207
BGN	NM_001711.3	2.20144
IGFBP6	NM_002178.2	2.260575
FSTL1	NM_007085.3	2.318603
ADAM19	NM_033274.2	2.343654
FBLN1	NM_006487.2	2.418595
F3	NM_001993.2	2.446623
COL11A1	NM_080629.2	2.50218
SERPINF1	NM_002615.4	2.655044
FBLN2	NM_001998.2	2.798432
EFEMP2	NM_016938.2	2.833782
LOXL3	NM_032603.2	3.072924
FN1	NM_212474.1	3.253216
SFRP2	NM_003013.2	3.32327
COL5A2	NM_000393.3	3.63136
COL1A2	NM_000089.3	3.631591
COL1A1	NM_000088.3	4.273946
TGFBI	NM_000358.1	4.396439
POSTN	NM_006475.1	5.025622
DKK3	NM_013253.4	5.166207
COL5A1	NM_000093.3	5.221677
SPARC	NM_003118.2	5.568203
GREM1	NM_013372.5	5.671927
MFGE8	NM_005928.1	5.955669

combination with increased proliferation of chondrocytes, is likely responsible for the relative overgrowth of chondrocytes after 4 weeks of coculture. This is particularly evident in the SVF coculture group compared to the ASC coculture group and was confirmed by STR analysis demonstrating that the initial seeding ratio of CH: SVF/ASC changed from 1:4 to 3:2 in case of coculture with SVF and 1:3 in case of ASC. We cannot rule out the possibility that some chondrocytes also died in coculture pellets. However, STR analysis in combination with the positive TUNEL staining suggests that predominantly the SVF, and to a lesser extent ASC, had disappeared from the cocultures by cell death.

Based on our results, SVF cells could be a very good, relatively easily accessible, and abundant source of cells to be used for cartilage regeneration. In previous studies, Jurgens *et al.* reported that SVF cells showed stem cell characteristics that are very similar to expanded cells isolated

from SVF, and SVF cells even appeared to be slightly better than expanded cells isolated from SVF in chondrogenic differentiation as indicated by GAG quantification.<sup>15</sup> In comparison with bone marrow-derived MSCs, SVF cells also differentiate into osteogenic, adipogenic, and chondrogenic lineages, but have a much higher colony-forming unit (CFU) frequency.<sup>42</sup> It has also been reported that SVF cells could quickly attach to poly (L-lactide-co-caprolactone) and collagen type 1/3 scaffolds, both of which are suitable for cartilage tissue engineering.<sup>43</sup>

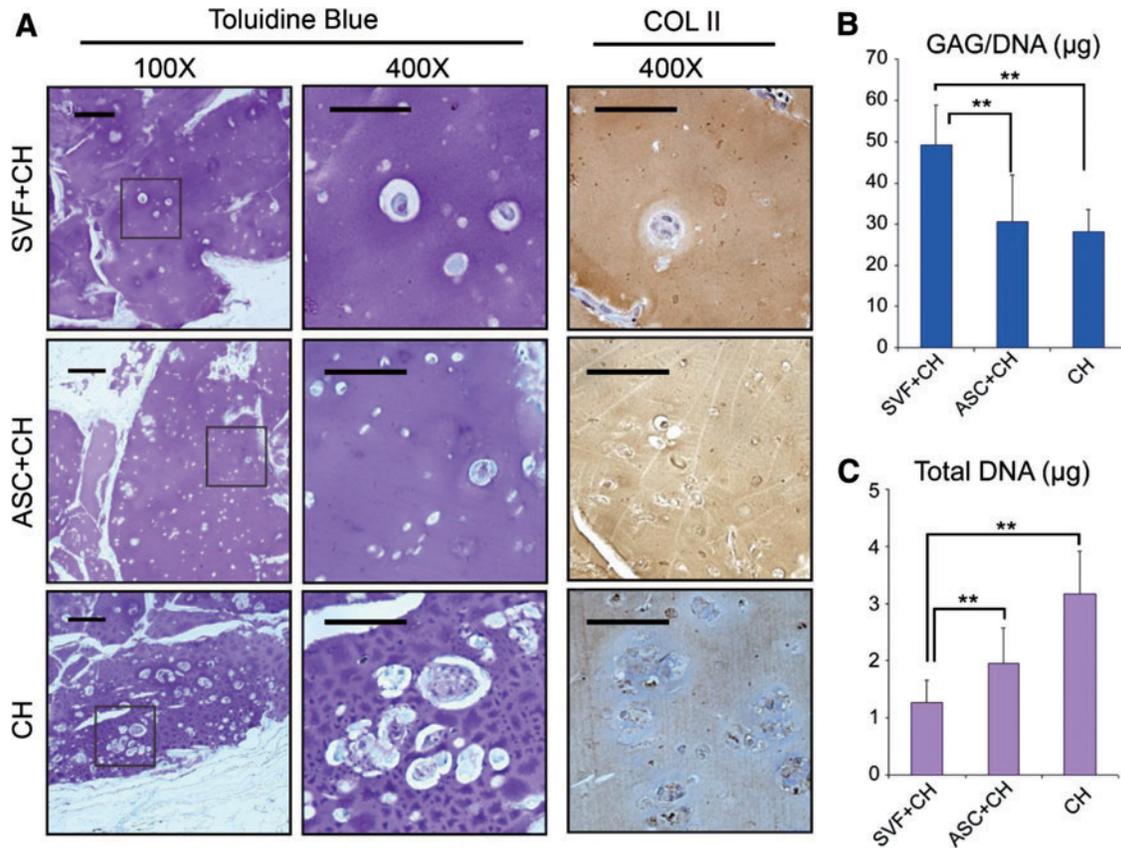
In this study, we show data to support that cocultured pellets of SVF cells and chondrocytes deposit more cartilage matrix than cocultured pellets of ASC and chondrocytes as well as monoculture of chondrocytes. Even after coimplantation of SVF or ASC in an alginate gel, more ectopic cartilage matrix formation is observed in the combination of SVF with chondrocytes after subcutaneous implantation in a nude mouse model. Improved cartilage formation in combination of SVF with CH was most clearly seen in the increased GAG deposition both *in vitro* and *in vivo*. It was less clear based on collagen type 2 deposition, in which the CH group displayed the strongest staining. This suggests that GAG and collagen type 2 deposition were independently regulated by coculture of CH with SVF.

Nevertheless, the superiority of the SVF chondrocyte combination over chondrocytes alone is still obvious for a practical reason, particularly if we take into consideration that the number of chondrocytes needed for coimplantation (SVF and chondrocytes) is much lower than that needed for a single implantation (chondrocytes alone). Since SVF can be relatively easy and quickly isolated out of a liposuction, it is possible to isolate SVF and reimplant the cells into the patient in a one-step surgery.<sup>44,45</sup>

In marked contrast to ASC, SVF contains a heterogeneous cell population.<sup>46</sup> It is believed that ASC have a more homogeneous composition of cell types, even though ASC and SVF share some common features like multilineage differentiation. In addition to this, our data from FACs and microarray indicated that ASC resemble more mesenchymal cell lineages than SVF. This is in agreement with literature indicating that the SVF contains endothelial cell and hematopoietic cell populations besides a MSC fraction.<sup>42</sup> With unknown mechanism, these nonmesenchymal cells enhanced the trophic effects of MSCs at least in coculture with chondrocytes.

There could be two hypotheses to explain this mechanism: (1) one or more of the non-MSC populations are actually better trophic mediators enhancing matrix formation of chondrocytes than MSCs; (2) some of the non-MSCs preserve/increase the trophic effects of MSCs. So far, we do not have enough data to exclude one of the above possibilities. However, pre-existing data seem to support the second. It has been reported that ASC resided in the adventitia of blood vessels as a group of CD34<sup>+</sup>/CD31<sup>-</sup>/ $\alpha$ -smooth muscle actin- (smA) cells.<sup>47-49</sup> Our previous data also demonstrated that a small fraction of CD146-positive cells were present in both SVF and ASC.<sup>14,15</sup>

There are debates about the CD markers of these cells<sup>50,51</sup>; nevertheless, all these studies pointed out that ASC reside in a perivascular niche *in situ*. This niche or local microenvironment dominantly made of endothelial cells is important in maintaining the ASC phenotype as a



**FIG. 5.** Coimplantation of chondrocytes with SVF produces more GAG than with ASC. **(A)** SVF or ASC from three donors was pooled to eliminate donor variations, as well as chondrocytes from three donors. Then SVF or ASC were coimplanted with chondrocytes at a ratio of 4:1 into nude mice for 8 weeks. Constructs with chondrocyte only were served as positive control, while constructs without cells (blank) were used as negative control. Toluidine blue staining indicates the presence of GAGs. Immunohistochemical staining shows the expression of collagen type II. Scale Bar = 100 µm. Right panel shows the enlarged images of the inserts in left panel. **(B)** GAG normalized to DNA in constructs. Amount of GAG and DNA of constructs ( $N=8$ ) were measured by GAG and DNA assay. Values were subtracted by blank constructs to eliminate the influence of material on GAG assay. Double asterisks represent  $p < 0.01$ . NS = Not Significant. Error bar reflects SD. **(C)** Total DNA in TE constructs. Values were subtracted by blank constructs (without any cells) to eliminate the influence of material on GAG assay. Asterisk represents  $p < 0.05$ . Double asterisks represent  $p < 0.01$ . NS = Not Significant. Error bar reflects SD. Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

stem cell. Although speculative, it may also be crucial for keeping the adipose stem cell's function as a trophic mediator. It was believed that ASC could stabilize endothelial networks *in vitro* and help vessel formation *in vivo*.<sup>52</sup> As a feedback, it is also possible that endothelial cells may better preserve some of the adipose stem cell's characteristics which are lost during *in vitro* expansion, such as their ability to express trophic mediators.

Regarding the interaction between different cell populations in SVF, one possible interaction that we should keep in mind is the one between hematopoietic cells and MSCs. It was reported that MSCs could support hematopoiesis in culture.<sup>53</sup> In turn, hematopoietic cells such as monocytes and lymphocytes, which are abundant in SVF but absent after *in vitro* expansion, may also regulate the trophic property of MSCs.

In conclusion, our data demonstrated that chondrocytes form better cartilage tissue when cocultured or coimplanted with SVF of adipose tissue than with ASC. Heterogeneous

populations of SVF show higher level of trophic effects in cartilage formation. These results support the clinical potential of a one-step therapy for cartilage repair, in which SVF from adipose tissue and chondrocytes from the nonweight bearing joint surface are isolated, mixed, and implanted back into the patient during the same surgical procedure.

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#### Disclosure Statement

No competing financial interests exist.

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