TISSUE ENGINEERING: Part A
Volume 20, Numbers 15 and 16, 2014

DOI: 10.1089/ten.tea.2013.0559

Serum- and Growth-Factor-Free Three-Dimensional Culture System Supports Cartilage Tissue Formation by Promoting Collagen Synthesis via Sox9–*Col2a1* Interaction

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Objective: One of the factors preventing clinical application of regenerative medicine to degenerative cartilage diseases is a suitable source of cells. Chondrocytes, the only cell type of cartilage, grown *in vitro* under culture conditions to expand cell numbers lose their phenotype along with the ability to generate hyaline cartilaginous tissue. In this study we determine that a serum- and growth-factor-free three-dimensional (3D) culture system restores the ability of the passaged chondrocytes to form cartilage tissue *in vitro*, a process that involves sox9.
Methods: Bovine articular chondrocytes were passaged twice to allow for cell number expansion (P2) and cultured at high density on 3D collagen-type-II-coated membranes in high glucose content media supplemented with insulin and dexamethasone (SF3D). The cells were characterized after monolayer expansion and following 3D culture by flow cytometry, gene expression, and histology. The early changes in signaling transduction pathways during redifferentiation were characterized.
Results: The P2 cells showed a progenitor-like antigen profile of 99% $CD44^+$ and 40% $CD105^+$ and a gene expression profile suggestive of interzone cells. P2 in SF3D expressed chondrogenic genes and accumulated extracellular matrix. Downregulating insulin receptor (IR) with HNMPA-(AM3) or the PI-3/AKT kinase pathway (activated by insulin treatment) with Wortmannin inhibited collagen synthesis. HNMPA-(AM3) reduced expression of *Col2*, *Col11*, and *IR* genes as well as *Sox6* and *-9*. Co-immunoprecipitation and chromatin immunoprecipitation analyses of HNMPA-(AM3)-treated cells showed binding of the coactivators Sox6 and Med12 with Sox9 but reduced Sox9–*Col2a1* binding.
Conclusions: We describe a novel culture method that allows for increase in the number of chondrocytes and promotes hyaline-like cartilage tissue formation in part by insulin-mediated Sox9–*Col2a1* binding. The suitability of the tissue generated via this approach for use in joint repair needs to be examined *in vivo*.

Introduction

Development of new biological treatments for cartilage degradation has been hampered by lack of sufficient numbers of cells that exhibit the appropriate phenotype of articular chondrocytes. The most commonly used method to increase the number of cells is to culture them in monolayer (ML) *in vitro*. However, under these conditions the chondrocyte phenotype changes; cells obtain a spindled morphology and lose their chondrocytic characteristics resulting in an inability to form cartilage tissue *in vitro* unless manipulated in some way such as coculture with differentiated chondrocytes.[1,2] Use of passaged chondrocytes for cartilage repair is FDA approved and has been utilized clinically for autologous chondrocyte transplant for more than 10 years, although with limited success in part because of their inability to form articular cartilage *in vivo*.[3] Therefore, developing a method that uses passaged chondrocytes to generate hyaline cartilage without any supplementation is particularly attractive as it could shorten the time to clinical translation.

It has been suggested that, with passage, as chondrocytes lose their phenotype they acquire some characteristics of mesenchymal stromal cells (MSCs) as defined by spindled morphology; higher *collagen type I* gene expression; cell

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surface profile of $CD105^+$, $CD90^+$, $CD73^+$, $CD44^+$, $CD45^-$, and $CD34^-$; and the ability to differentiate to tissues of mesenchymal lineage.[4–7] A number of conditions have been used to redifferentiate these passaged cells to chondrocytes, such as coculture or three-dimensional (3D) culture.[8,9] All of these approaches require the presence of fetal bovine serum (FBS), exogenous hormones, and/or growth factors of the transforming growth factor (TGF)β family—conditions that can trigger hypertrophic differentiation or fibrocartilage formation.[10,11] Proteomic analysis shows that FBS contains fibronectin, collagen type 2, and collagen type 1 that may promote cell attachment. It also contains growth factors, such as FGF, TGFβ1, glial growth factor, and prepro-insulin-like growth factor 1 (PIlGF—signal peptide containing IGF-1, functionally similar to insulin), in lot dependent concentrations.[12] Hence, with FBS supplementation reproducibility becomes dependent on the batch of serum. The xenogeneic nature of FBS is also a concern for tissue engineering both in terms of the potential for inducing an immune response and/or transmitting disease.[13] The serum substitute ITS+ (insulin, transferrin, selenium, and dexamethasone; BD Bioscience) is often used to circumvent these limitations and is commonly used together with TGFβ to differentiate MSCs to chondrocytes. This may be due in part to the presence of insulin, a hormone highly conserved among vertebrates. It is a component of ITS+ and is known to have effects on cell survival, proliferation, differentiation, metabolism, and during development.[14] Insulin binds to the insulin receptor (IR) that activates cytoplasmic substrates insulin receptor substrate 1 (IRS-1) and/or Shc that in turn activate ERK-MAPK, PI-3 kinase, and/or Akt signal transduction pathways.[15] These pathways have been implicated in regulating chondrogenesis, although Akt activation has been shown to favor chondrocyte hypertrophy.[16] Importantly chondrocytes express functional IRs and levels may be altered in osteoarthritic chondrocytes.[17] Recently insulin has been shown to regulate Sox9 expression in mesenchymal cells.[18] Sox9, the master transcription factor regulating chondrogenesis, is expressed at the earliest stage of cartilage anlagen formation by chondrocytes.[19] Sox9 requires additional coactivators, such as Sox5, Sox6, Med12, and SP1, to form a functional transcriptional complex[20,21] that regulates gene expression of many cartilage-related molecules, including *Col2a1*, *Col9a2*, *Col11a2*, *aggrecan*, and cartilage oligomeric matrix protein (*COMP*).[20,22–24]

This study demonstrates that in the presence of media supplemented with ITS+ instead of FBS, passaged bovine chondrocytes when grown on collagen-type-II-coated membrane inserts revert back to articular chondrocytes that form hyaline cartilage tissue. The insulin in the culture media contributes to the ability of the redifferentiating cells to form cartilage tissue by sox9-mediated collagen II gene and protein expression. Understanding the mechanism(s) regulating redifferentiation will allow identification of the conditions that will support this redifferentiation process in human chondrocytes.

Materials and Methods

Cell isolation and culture

Bovine articular chondrocytes (BACs) were harvested by enzymatic digestion from cartilage obtained from bovine metacarpo-phalangeal joints (6–9 months old) as described previously.[19] BACs (2000 cells/cm^2) were cultured in ML in 5% FBS and passaged twice (P2) to attain up to 200-fold increase in cell number. P2 (1.5×10^6 cells) were seeded onto type-II-collagen-coated Millicell® culture inserts (60 mm^2; Millipore) and cultured for up to 4 weeks in serum-free 3D culture (SF3D) consisting of high glucose content medium (HG) Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L), ITS+ (10 μg/mL insulin, 0.5 μg/mL transferrin, 0.67 ng/mL selenium, 5.35 μg/mL linoleic acid, and 1.25 mg/mL BSA; BD Bioscience, MA), proline (40 μg/mL), pyruvate (110 μg/mL), dexamethasone (0.1 μM), and ascorbate-2-PO_4 (50 μg/mL). As controls, cells were grown in serum-containing 3D culture (SC3D) with 20% FBS.

Inhibition studies

Twenty-four hours after seeding, the P2 cells were serum or ITS+ starved for 18 h and treated with the IR inhibitor HNMPA-(AM3) (100 μM; Enzo Life Sciences)[25] and PI-3 kinase (upstream of AKT) inhibitors Wortmannin (5 ng/mL; Sigma-Aldrich) or LY294002 (10 μM; Millipore). Insulin was added after 2 h and tissue was harvested after 24 h. Controls were cultures that received carrier (DMSO) only.

Flow cytometry

Cells were harvested using trypsin (1× for 5 min), allowed to recover for 30 min in 2% FBS containing phosphate-buffered saline (PBS), and stained with either CD105-PE or CD44-PE (12-1057 and 12-0441; eBioscience). Cells were analyzed by EPICS XL FACS and Kaluza analysis software (Beckman Coulter).

Histology and immunohistochemistry

Tissues were fixed in 10% formalin and embedded in paraffin, and 5-μm sections were cut. Representative sections were stained with toluidine blue. For immunohistochemistry deparaffinized sections were digested at RT for 10 min with 0.4% pepsin (w/v) (Sigma-Aldrich) in TBS-HCl (pH 2.0), blocked with 20% goat serum (v/v) (Sigma-Aldrich), and incubated overnight at 4°C with either type I collagen (1:100, T59103R; Meridian) or type II collagen (1:100, MAB8887; Millipore) antibody. Immunoreactivity was detected by Alexa-488 goat anti-rabbit or Alex-594 goat anti-mouse secondary antibody (1:300, 1 h; Invitrogen). Nuclei were visualized by DAPI (1:10,000; Invitrogen). IgG was used as the negative control. Images were collected using a 40× objective (Nikon Eclipse C1si).

Tissue analysis

The tissues were digested in papain (40 μg/mL in 20 mM ammonium acetate, 1 mM ethylenediaminetetraacetic acid [EDTA], and 2 mM DTT; Sigma-Aldrich) for 48 h at 65°C and assayed as described previously.[2] Briefly, DNA content was quantified by Hoechst dye 33258 assay (Polysciences, Inc.) and fluorometry (excitation $\lambda = 365$ nm and emission $\lambda = 458$ nm). Proteoglycan (PG) content was estimated by the dimethylmethylene blue dye binding assay ($\lambda = 525$ nm) (Polysciences, Inc.). Collagen content was quantified by chloramine-T/Ehrlich's reagent assay ($\lambda = 560$ nm).

Table 1. List of Sequences of Primers Used in Quantitative PCR

Gene	*Forward (5′)*	*Reverse (5′)*
Sox6	tcctggcagcgcatgatgaaca	agcaaggtccatttgctgccgt
Sox9	acacacagctcactcgaccttg	agggaattctggttggtcctct
Col1a1	cggctcctgctcctctac	cacacgtctctctcggtcatggta
Col2a1	gtgtcacggccaggatgtc	gcagaggacagtcccagtgt
Col9a1	aacgacggaggccttacagacgg	aattcctggagaaccttgcgg
Col10al	tccaaaatacaggtctgagc	cctgttaattgtcagaacag
Col11al	acagttgtgagtgcgggggct	tcccagagccaccgtttcgt
TenacinC	aagtcatccggcacaagcagca	acgtgattgaacaccaccggct
Wnt9a	acaacctcgtgggtgtgaaggt	tcgtacttgtgcttcaggcgct
Gdf5	tggtgtttggccgcaccaagaa	aagttgacatgcagcgccttcc
Gli3	tcggccagatgtcagcgagaaa	tgtggctgcatagtgactgcgt
Aggrecan	tgggactgaagttcttggaga	gcgagttgtcatggtctgaa
COMP	tgcctgtgacaactgtcctcagaa	ttgtctaccaccttgtccgcatca
CD44	gatccacccccaattccatctgt	aaccgcgagaatcaaagccaa
IR	tccgcacactcggtttctcctt	agggttcaaaccttgcagggca
IGF1R	ccgagttcccagagctgtgcagtta	gatggtccacactgggcaagacc

PCR, polymerase chain reaction.

RNA isolation and relative quantitative PCR

Total RNA was extracted with RNeasy® kit (Qiagen) and reverse transcribed with 200 units of SuperscriptIII (Invitrogen). SYBR green dye I, 0.2 μM of primers (Table 1), and realplex2 Master cycler (Eppendorf) were used for relative quantitative PCR (qPCR).

Radiolabeling

Synthesis of collagens and PGs was evaluated by assaying the incorporation of [^{3}H]-proline and [^{35}S]-SO_4 (2 μCi; PerkinElmer), respectively, 3 days after cell seeding. Cells were labeled for 24 h, washed in PBS, and digested in papain as described previously. Collagen and PG were precipitated from the media with 70% ammonium sulfate or 100% cold ethanol, respectively, and centrifuged at 14,000 rpm for 30 min at 4°C. Pellets were washed with cold 70% ethanol, and centrifuged for 10 min at 14,000 rpm. The precipitated collagen was resuspended in 10% sodium dodecyl sulfate (SDS) and the PGs were solubilized in 4 M guanidine hydrochloride and quantified using a β-scintillation counter (Beckman Coulter).

To determine glucose uptake, cells were cultured in SF3D-HG, SF3D-low glucose content media (LG), or SC3D-HG for 24 h; serum or ITS and glucose starved for 2 h; and then incubated with [^{3}H]-deoxyglucose (2D-G ^{3}H; 10 μCi/mL) for 30 min at 37°C. The cultures were rinsed with PBS, and digested with papain (40 μg/mL) for 24 h. Glucose uptake was quantified by measuring ^{3}H incorporation using a β-scintillation counter and normalized to DNA content.

Immunoblotting

Cells were harvested in RIPA buffer containing 1% NP40, 0.1% SDS, 2 mM EDTA, 0.5% sodium deoxycholate (NaDC), and 150 mM NaCl in 50 mM Tris (pH 7.8). Fifteen micrograms of protein was separated on 12% SDS–polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane by iblot (Invitrogen). Membranes were blocked in 1% milk for 1 h and incubated with antibodies reactive with Sox9 (1:1000, ab3697; Abcam), β-actin (1:2000, A5441; Sigma-Aldrich), Shc, pSHC, pAKT, AKT, pERK, or ERK (1:1000, respectively, 2432, 2434, 9611s, 9272, 9102, and 9101; Cell Signalling) overnight. Washed membranes were incubated with HRP-conjugated secondary antibodies for 1 h and immunoreactivity was detected by ECL+ (GE Healthcare).

Co-immunoprecipitation

Lysis buffer containing 1% Triton X-100, 10% glycerol, 137 mM NaCl, 1 mM Na-orthovanadate, and 1× protease inhibitors (Complete mini; Roche) was used to extract protein. Cell lysates (120 μg) were centrifuged at 15,000 *g* for 20 min and the supernatant was precleared with protein A/G beads (Millipore) for 2 h and then incubated for 4 h with antibody reactive with Sox9 (1:1000, ab3697; Abcam) at 4°C. The immune complex was harvested by incubation with Protein A/G beads overnight at 4°C. Beads were washed in lysis buffer and boiled for 10 min in mercaptoethanol containing sample buffer and immunoblotted (see immunoblotting section) with antibodies reactive with Sox6 and Med12 (1:1000, ab66316 and ab49053; Abcam).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP)–qPCR analysis was carried out using genomic DNA. Briefly, 2-day-old cultures were crosslinked *in situ* with 0.75% formaldehyde and harvested in 50 μL/10^6 cells of lysis buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 1% SDS, and 0.1% NaDC). DNA was sheared by sonication at 25% amp (30 s ON and 60 s OFF 20×; Vibracell) to 250–900 bp fragments (confirmed by agarose gel); a fraction was used as input control. Twenty-five micrograms of DNA was diluted 1:10 in RIPA and immunoprecipitated with antibody reactive with Sox9 and Protein A/G beads overnight at 4°C. Samples were reverse crosslinked in the presence of 5 μL Proteinase K at 65°C overnight. Co-immunoprecipitated DNA was purified with equal volume of 1:1 phenol:chloroform and washed with 3× volume of ethanol containing 10 μL of glycogen (5 mg/mL). The air-dried pellet was resuspended in 100 μL of water; qPCR with *Col2a1* primers (f-5′ TTCCAGATGGGG CTGAAACGCT, r-5′ TGGGGCTTTTCTCGAGCACACA) located in the Sox9 binding region was carried out (see Table 1).

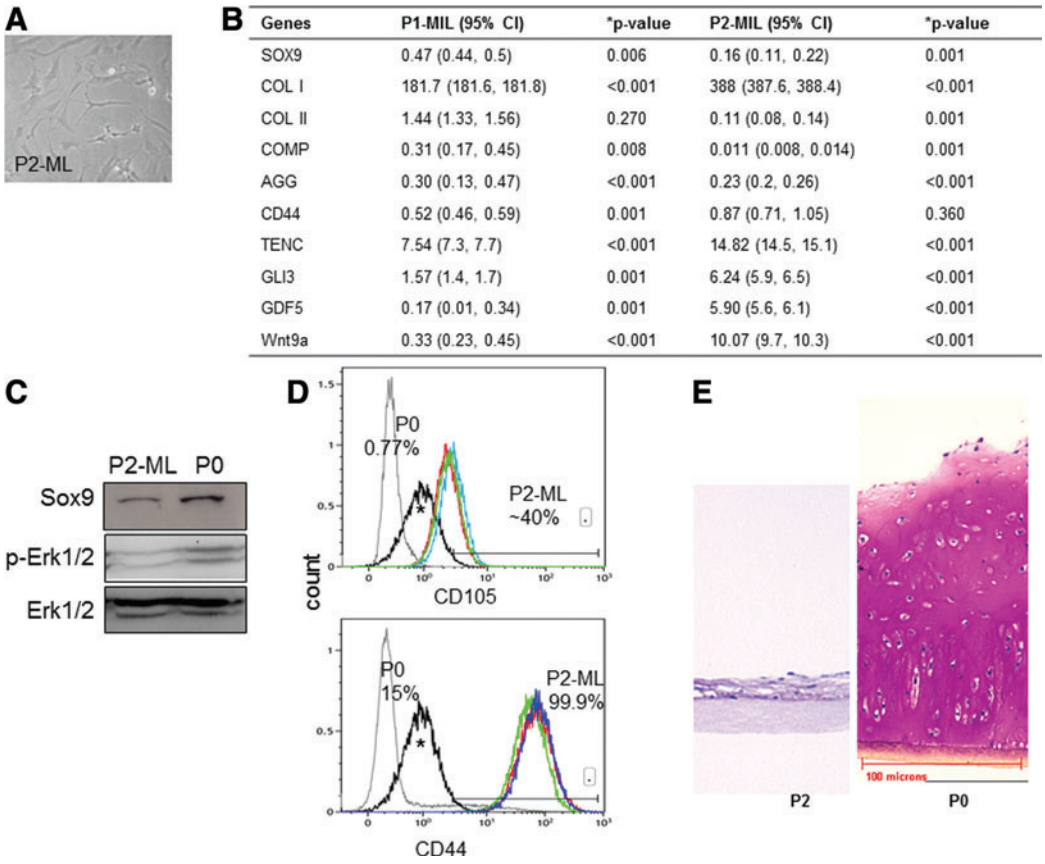
Statistical analysis

Each experiment was done using cartilage tissues obtained from a single animal. The results are expressed as the mean of three to five independent experiments. Each condition was done in triplicate. Pearson's chi-square test was used to verify the Gaussian distribution and independence of the data. One-way analysis of variance followed by Tukey's *post hoc* test was used for all pair-wise comparisons between groups. *p*-Values ≤ 0.05 were considered to be statistically significant. Data are presented as mean with 95% confidence interval (95% CI).

Results

Passaged chondrocytes lose their chondrocytic phenotype and do not form cartilage tissue in 3D culture in the presence of serum containing media

Cell passaging in ML culture resulted in an increase in cell number but cells lost their polygonal shape and acquired a spindled morphology (Fig. 1A). Gene expression analysis after each passage showed progressive loss of *Col2a1*, *Sox9*, *aggrecan*, and *COMP*, and gain of *Col1a1* expression. In addition



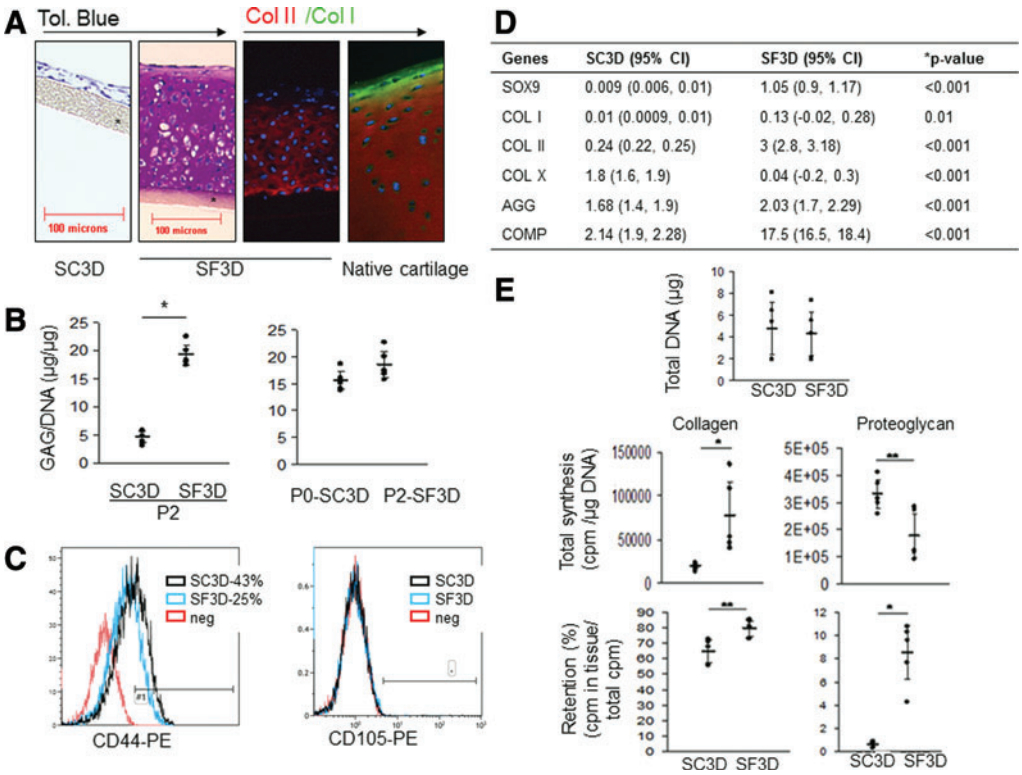
Genes	P1-MIL (95% CI)	*p-value	P2-MIL (95% CI)	*p-value
SOX9	0.47 (0.44, 0.5)	0.006	0.16 (0.11, 0.22)	0.001
COL I	181.7 (181.6, 181.8)	<0.001	388 (387.6, 388.4)	<0.001
COL II	1.44 (1.33, 1.56)	0.270	0.11 (0.08, 0.14)	0.001
COMP	0.31 (0.17, 0.45)	0.008	0.011 (0.008, 0.014)	0.001
AGG	0.30 (0.13, 0.47)	<0.001	0.23 (0.2, 0.26)	<0.001
CD44	0.52 (0.46, 0.59)	0.001	0.87 (0.71, 1.05)	0.360
TENC	7.54 (7.3, 7.7)	<0.001	14.82 (14.5, 15.1)	<0.001
GLI3	1.57 (1.4, 1.7)	0.001	6.24 (5.9, 6.5)	<0.001
GDF5	0.17 (0.01, 0.34)	0.001	5.90 (5.6, 6.1)	<0.001
Wnt9a	0.33 (0.23, 0.45)	<0.001	10.07 (9.7, 10.3)	<0.001

FIG. 1. Loss of chondrocytic phenotype in culture-expanded cells. **(A)** Spindle-shaped morphology of P2 cells in monolayer (ML). $n=5$ experiments. **(B)** Gene expression profile of cells after each passage compared with P0 levels. A significant increase in *Col1a1* was observed after the first passage along with downregulation of cartilage-associated gene *aggrecan* and cartilage oligomeric matrix protein (*COMP*) whereas a decrease in *Col2a1* was seen only after the second passage. Genes expressed by interzone cells—*TenacinC*, *Gli3*, *GDF5*, and *Wnt9a*—were significantly upregulated in P2 cells compared with P0 or P1 cells. Data are expressed as mean with the uncertainty estimated by 95% confidence interval (95% CI; lower and upper limits are within brackets). $n=5$ experiments. P0, primary chondrocytes; P1-ML or P2-ML, passage 1 or 2 cells, respectively, harvested from ML. **(C)** Immunoblot analysis showed a decrease in Sox9 and p-ERK-1/2 levels in P2-ML compared with P0. $n=3$ experiments. **(D)** FACS analysis of surface markers showed 40% $CD105^+$ and 99% $CD44^+$ cells after two passages. $n=3$ experiments. ^Nonspecific background staining in P2 cells. **(E)** Photomicrographs of toluidine-blue-stained tissues formed by P2 cells cultured in serum containing three-dimensional conditions (SC3D) for 4 weeks show loss of capacity to accumulate cartilaginous matrix by P2 cells unlike P0 that retained abundant proteoglycan (PG)–rich matrix. $n=5$ experiments. ¶Collagen-type-II-coated membrane insert. Color images available online at www.liebertpub.com/tea

the cells showed a significant increase in the expression of interzone cell markers—*Gli3*, *tenascinC*, *Gdf5*, and *Wnt9a* (Fig. 1B). Sox9 and p-ERK-1/2 protein levels were decreased in passaged cells (P2) as determined by western blot analysis (Fig. 1C). Flow cytometry showed that >99% of P2 cells expressed the hyaluronan receptor $CD44^+$ and nearly 40% of the cells were $CD105^+$, a marker of marrow stromal cells (Fig. 1D). The P2 cells were placed in SC3D, and histological analysis after 4 weeks showed that they were unable to form cartilage tissue, in contrast to the PG-rich tissue formed by the primary (P0) chondrocytes under the same conditions (Fig. 1E).

Passaged chondrocytes undergo phenotype reversal and form cartilage tissue in 3D culture in serum-free media

In SF3D, P2 reacquired the ability to form cartilage tissue as compared with cells grown in the presence of serum. After 4 weeks of culture P2 had accumulated abundant hyaline cartilaginous extracellular matrix that contained sulfated PGs (Fig. 2A) and type II collagen. Type I collagen was not detected by immunostaining (Fig. 2A). The amount of glycosaminoglycans was similar to that accumulated by P0 grown in SC3D culture (Fig. 2B). FACS analysis of the P2 at 48 h of SF3D culture revealed that only 25% of P2 in SF3D were $CD44^+$ and 100% had lost CD105 antigen (Fig. 2C). Gene expression analysis at 3 weeks showed that *Col2a1*, *Sox9*, *aggrecan*, and *COMP* were higher in SF3D compared with P2 cells grown in SC3D. *Col1a1* gene expression was lower in both conditions. *Col10a1* was significantly lower in SF3D (Fig. 2D). *Col10a1* levels were in the same range as that found in primary chondrocytes (data not shown). Cells grown under either culture conditions showed similar DNA content (Fig. 2E). Analysis of $[^3H]$-proline and $[^{35}S]$-SO_4 incorporation at day 2 of culture showed that P2 in SF3D synthesized and retained significantly more collagens. PG synthesis was higher in the SC3D culture conditions; however, significantly more PGs were retained in SF3D (Fig. 2E). This suggested that collagen synthesis and retention was important for



Genes	SC3D (95% CI)	SF3D (95% CI)	*p-value
SOX9	0.009 (0.006, 0.01)	1.05 (0.9, 1.17)	<0.001
COL I	0.01 (0.0009, 0.01)	0.13 (-0.02, 0.28)	0.01
COL II	0.24 (0.22, 0.25)	3 (2.8, 3.18)	<0.001
COL X	1.8 (1.6, 1.9)	0.04 (-0.2, 0.3)	<0.001
AGG	1.68 (1.4, 1.9)	2.03 (1.7, 2.29)	<0.001
COMP	2.14 (1.9, 2.28)	17.5 (16.5, 18.4)	<0.001

FIG. 2. Assessment of the tissue formed by the P2 cells in SF3D. **(A)** Photomicrographs of 4-week-old tissue formed by P2 cells cultured in SC3D (serum containing media) show no matrix accumulation (far left panel) while growth in SF3D (serum-free media) shows accumulation of matrix rich in PGs (toluidine blue; second panel). SF3D-generated tissue stained positively for collagen type II (red) and was predominantly negative for collagen type I (green) (third panel) similar to native cartilage tissue stained with the same antibodies (fourth panel). $n=5$ experiments. **(B)** More PGs (glycosaminoglycan) were accumulated in tissues formed by P2 in SF3D than in SC3D (left) and was comparable to that accumulated by P0 cells in SC3D (right) at 4 weeks of culture. Each dot represents an independent experiment; horizontal lines represent mean and 95% CI. $n=5$ experiments. **(C)** FACS analysis after 2 days of culture shows complete loss of CD105 in both the conditions while only 25% of the cells retained CD44 in SF3D in comparison to 43% in SC3D. $n=3$ experiments. **(D)** After 3 weeks the gene expression profile relative to freshly harvested P2-ML cells also shows that P2 in SF3D expressed significantly higher cartilage-related genes and lower *Col10a1* expression compared with the cells in SC3D. Data are shown as mean with the uncertainty estimated by 95% CI (lower and upper limits are within brackets). $n=3$ experiments. **(E)** Total DNA content of tissues was similar between the conditions. At 48 h, P2 in SF3D synthesized and retained more collagen and, although PG retention was higher in SF3D, more was synthesized in SC3D. ¶ Collagen-type-II-coated membrane inserts; all images are of the same magnification. Each dot represents the mean of an independent experiment; horizontal lines represent mean and 95% CI. $n=5$ experiments. $*p \le 0.01$, $**p \le 0.05$ compared with SC3D. Color images available online at www.liebertpub.com/tea

tissue formation and this parameter was used as the marker for short-term assessments in subsequent experiments.

Redifferentiation is insulin, dexamethasone, and HG dependent

To evaluate the contribution of the individual media components, P2 cells were cultured under various media conditions and histological analysis at 4 weeks of culture showed that insulin, dexamethasone (synthetic glucocorticoid), and high glucose content DMEM were all essential for cartilage growth (Fig. 3). In contrast to the thin fibrous layer of tissue formed by the P2 cells grown in the absence of insulin or in low glucose, P2 cells grown in insulin and HG resulted in thick hyaline-like cartilage tissue rich in PGs. The presence of dexamethasone also contributed to cartilage formation but was insufficient in the absence of HG or insulin to support generation of this tissue (Fig. 3). IGF-1 could not replace insulin as no tissue formed in the presence of this growth factor, even when HG and dexamethasone were present. The serum substitute ITS+ was replaceable by insulin. DNA content was measured after 48 h of culture and was similar in both HG and LG culture conditions, indicating that there were no differences in cell attachment and survival (data not shown).

Characterization of cells during early redifferentiation

Redifferentiation was evident as early as 48 h as shown by upregulation of *Sox9* and *Col2a1* gene expression and downregulation of *Col1a1* (Fig. 4A). Western blot analysis of protein extracts from 48-h-old SF3D cultures shows increased phosphorylation of the insulin/IGF signaling molecules Shc and AKT (Fig. 4B). Based on these results the remaining analyses were done within 48 h. Quantification of

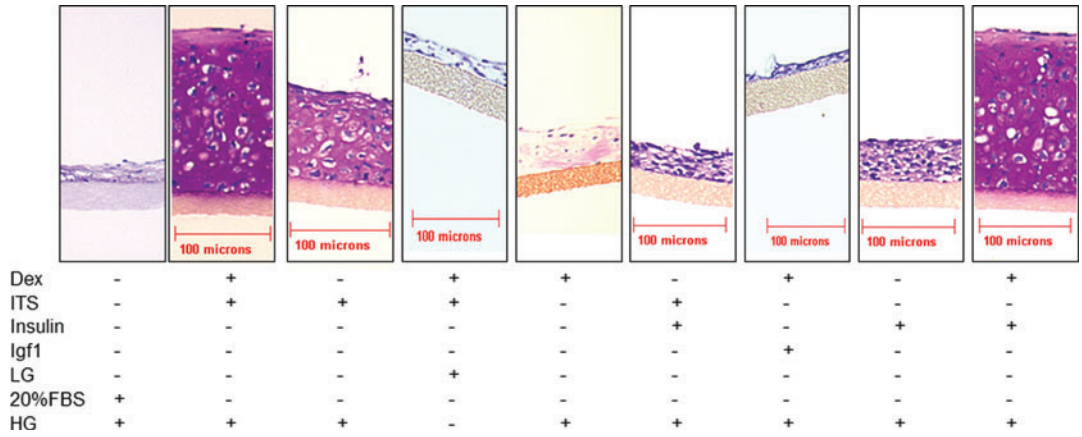


FIG. 3. Insulin, HG, and Dex are essential components of SF3D. Photomicrographs of the histological appearance of tissue formed by P2 under different media conditions after 4 weeks of culture. Dex, dexamethasone; HG, high glucose content media; LG, low glucose content media. ¶ Collagen-type-II-coated membrane inserts. $n = 3$ experiments. Color images available online at www.liebertpub.com/tea

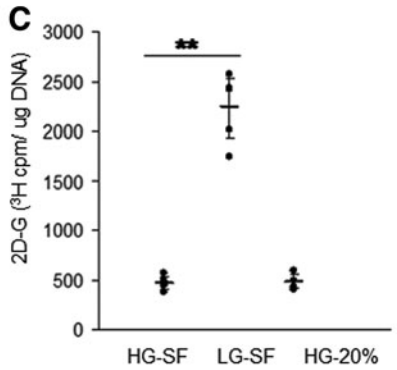
intracellular glucose levels showed that cells exhibited glucose uptake although those cultured in low-glucose media had significantly higher levels than in HG-SF or SC (20% FBS) condition (Fig. 4C).

Insulin signaling is involved in Sox9-mediated chondrogenic differentiation

The role of insulin in this process was evaluated using pharmacological agents known to inhibit insulin signaling. Synthesis of collagens was significantly reduced by HNMPA-(AM3) treatment, a drug known to inhibit the IR (Fig. 5A). PG synthesis was also inhibited by this treatment (data not shown). We confirmed by qPCR and immunoblotting that HNMPA-(AM3) treatment downregulated IR gene [control = 35 (33.9, 36) vs. HNMPA-(AM3) treated = 14 (13.4, 14.5; mean ± 95% CI)] and protein levels (Fig. 5B) and that the activity of Shc, a signaling pathway downstream of IR, was also reduced after IR inhibition (Fig. 5C). We then examined the levels of ERK/MAPK that lies downstream of Shc. Phosphorylation of 44-kDa ERK-1 subunit was unaffected after inhibition; however, the 42-kDa ERK-2 subunit was

A

Genes	24 hrs (95% CI)	P-value	48 hrs (95% CI)	p-value
SOX6	1.6 (1.5, 1.7)	0.048	4.1 (4.01, 4.19)	0.003
SOX9	4.5 (4.2, 4.8)	<0.001	3.6 (3.58, 3.62)	0.004
COL I	0.9 (0.2, 1.5)	0.314	0.9 (0.54, 1.26)	0.001
COL II	1.5 (1.45, 1.55)	0.024	7.1 (6.89, 7.31)	<0.001



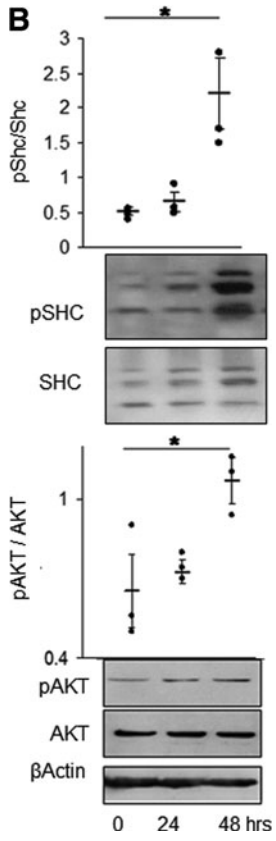


FIG. 4. Redifferentiation of P2 in SF3D. **(A)** Quantitative PCR analysis showed that cartilage-differentiation-associated genes in P2 cells were upregulated early. *Col1a1* was downregulated by 48 h of culture when compared with freshly harvested P2-ML cells. Data are shown as mean with 95% CI (lower and upper limits are within brackets). $n = 3$ experiments. **(B)** Representative immunoblots and densitometry of extracts from redifferentiating P2 cells in SF3D over the first 2 days of culture showed upregulation of insulin/IGF-pathway-related signaling molecules. **(C)** Glucose assay demonstrated higher uptake as indicated by increased intracellular glucose (2D-G ^{3}H) by P2 cells cultured under LG serum-free conditions. HG-SF, serum-free high glucose content medium containing ITS; LG-SF, low glucose content media containing ITS; HG-20%, high glucose content medium with 20% fetal bovine serum. Each dot represents the mean of an independent experiment; horizontal lines represent mean and 95% CI. $n = 5$ experiments. $^*p \le 0.01$, $^{**}p \le 0.05$.

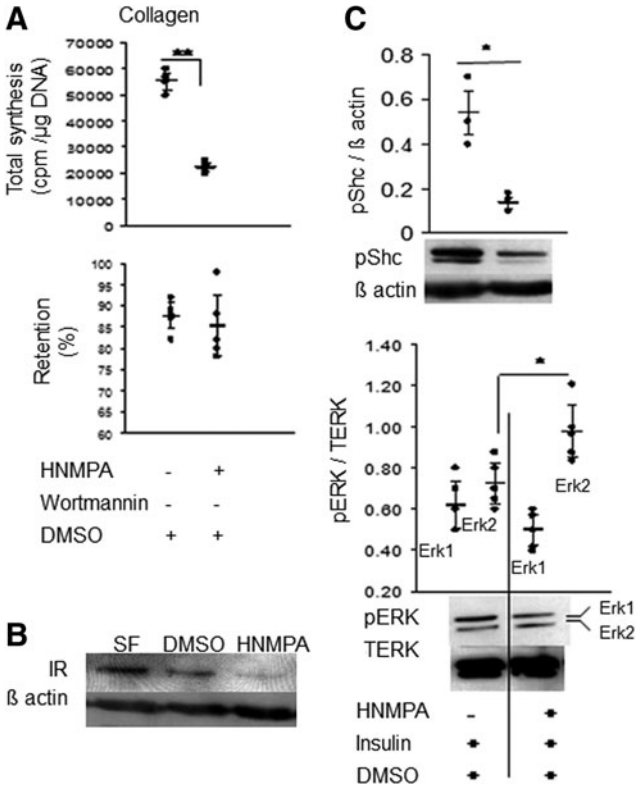


FIG. 5. Effect of inhibition of insulin receptor (IR). **(A)** Radioisotope incorporation studies show inhibition of total collagen synthesis but no change in % retention with HNMPA-(AM3) treatment. **(B)** Immunoblotting shows that HNMPA-(AM3) decreased IR protein levels. $n=3$ experiments. **(C, D)** Immunoblots followed by densitometry shows downregulation of Shc phosphorylation **(C)** and upregulation of 42-kDa ERK **(D)** in HNMP-treated cells. Each dot represents the mean of an independent experiment; horizontal lines represent mean and 95% CI. $n=3$ or 5 experiments. $*p \le 0.01$, $**p \le 0.05$ compared with DMSO control.

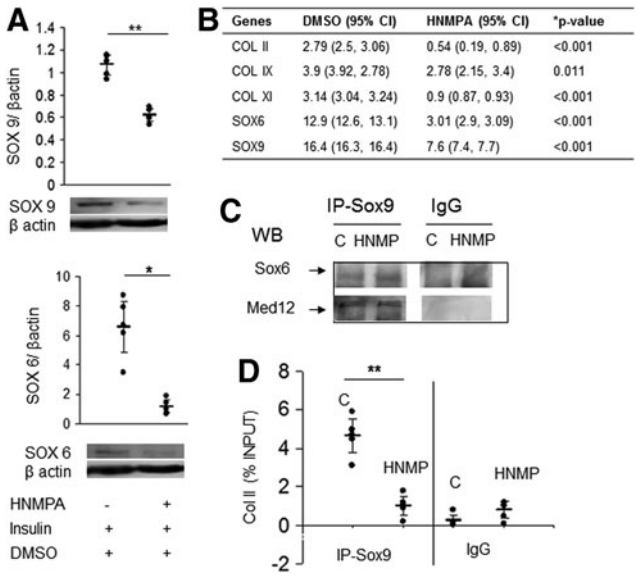


FIG. 6. Insulin mediates SOX9 levels and *col2a* expression. **(A)** Immunoblot and densitometry show that treatment with HNMPA-(AM3) leads to lower protein levels of Sox6 and Sox9 in P2 grown in SF3D. Each dot represents the mean of an independent experiment; horizontal lines represent mean and 95% CI. $n=5$ experiments. **(B)** Gene expression also shows decreases in cartilage-associated genes after downregulating IR. Data are expressed relative to P2-ML and are shown as mean with 95% CI (lower and upper limits). $*p \le 0.01$, $**p \le 0.05$. $n=3$ experiments. **(C)** Physical interaction of transcription factor Sox6 and binding protein MED12 with Sox9 is unaffected by HNMPA-(AM3) treatment as seen in co-IP analysis. $n=5$ independent experiments. **(D)** Chromatin immunoprecipitation extraction of Sox9-bound DNA showed decreased binding to *Col2a1* after HNMPA-(AM3) treatment (IR downregulation). Each dot represents the mean of an independent experiment; horizontal lines represent mean and 95% CI. $n=5$ experiments. $*p \le 0.01$. C, control; co-IP, co-immunoprecipitation.

significantly more phosphorylated, potentially implicating ERK-2 as a mediator of the insulin effect (Fig. 5D).

Both the gene and protein levels of sox9 and -6 were also significantly decreased by HNMPA-(AM3) treatment (Fig. 6). Gene expression analysis of P2 showed downregulation of cartilage-related collagens, *Col2a1* and *col11a1* (Fig. 6B). The Sox9 transcription complex likely remained unaffected as Sox6 and Med12 co-immunoprecipitated with Sox9 after HNMPA-(AM3) treatment (Fig. 6C). Given the decreased levels of Sox9 protein, we determined whether Sox9 binding to its target gene *Col2a1* was affected. ChIP analysis showed that Sox9 binds to the *Col2a1* gene at similar levels as in primary cells cultured in chondrogenic condition but the binding was significantly decreased in the treated P2 cells as would be expected given the decreased Sox9 levels (Fig. 6D). This suggested that insulin-regulated Sox9 levels influenced collagen synthesis by P2 cells.

Discussion

The data shows that chondrocytes serially passaged in ML culture (P2-ML) lost their chondrocyte phenotype and acquired a ''progenitor-like'' phenotype with a gene expression profile suggestive of interzone cells. These cells could be redifferentiated to chondrocytes that form hyaline-like cartilage tissue using a defined serum-free culture system (SF3D) in the absence of exogenous factors, such as TGFβ, as demonstrated by the ability of the cells to form cartilaginous hyaline tissue that appears qualitatively and quantitatively similar to the tissue formed by primary chondrocytes.[26] The redifferentiated cells showed elevated gene and protein levels of sox9 and mRNA levels of the cartilaginous matrix molecules *Col2a1*, *COMP*, and *aggrecan* and significantly lower *Col1a1* expression compared with P2-ML. Expression of *Col10* was low in SF3D and immunostaining of tissue sections also did not show collagen type 10 in tissue formed in SF3D at 3 weeks (data not shown). These findings suggest that the redifferentiated chondrocytes have an articular phenotype.[27] This redifferentiation in HG appears to be regulated in part by a signal transduction cascade involving insulin that upregulated Sox9 expression and modulated *col2a* expression and collagen synthesis. These findings are consistent with other studies that show that chondrocyte phenotype can be

changed by modulating the microenvironment and/or culture conditions.[10,26,28]

SF3D culture provides a sufficient chondro-conducive environment for the P2 cells that obviates the need for addition of exogenous TGFβ family molecules. Interestingly, SF3D does not invoke the same response (formation of cartilage tissue) in primary chondrocytes (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). To our knowledge, almost every study on redifferentiation of passaged chondrocytes has supplemented media with BMP, TGFβ, or some other type of growth factor.[11,29] In this SF3D system addition of TGFβ3 or inhibition of TGFβ3 signaling (SB431542) showed no effect on collagen synthesis by P2 cells further confirming the independence of this redifferentiation process on exogenous TGFβ (Supplementary Fig. S2) and that the cells were likely not producing TGFβ endogenously. It is possible that P2 cells in SF3D do not require exogenous growth factors, such as TGFβ, due to their unique progenitor-like phenotype with interzone features. A number of previous studies have also shown that passaged cells acquire some features of either MSCs[5,6,30] or interzone cells, such as GDF5 protein expression.[31] This type of differentiation may impart a unique phenotype on bovine P2 cells, which may explain their independence of growth factors. Additional studies are needed to further characterize this phenotype.

While type-II-collagen-coated membranes are part of the SF3D system, the critical components of the DMEM media are insulin, dexamethasone, and high glucose. Since all the three media components are essential for the tissue formation, it is difficult to determine whether they support each other or act independently. Glucose levels are important as culturing in low glucose prevented cartilage tissue formation. The mechanism by which glucose regulates redifferentiation and matrix accumulation is not clear; especially as in this system glucose uptake by the redifferentiating cells was greater in the presence of low-glucose media. HG levels have been shown to promote chondrogenesis in MSCs by downregulating ERK and upregulating p38. Glucose can have other effects such as inducing *O*-glycosylation of EGF domains in PGs[32,33] and macromolecular crosslinking that may alter matrix retention.[34] Further studies will be required to delineate the role of glucose in this system. It is highly likely that dexamethasone acted by influencing matrix synthesis because the tissue formed by the cells cultured without dexamethasone contained much less matrix compared with the cells grown in the presence of dexamethasone (Fig. 3A). In support of this, blocking the effect of dexamethasone by treating cells with the glucocorticoid receptor antagonist led to decreased collagen and PG synthesis (data not shown). Interestingly, glucocorticoids have been shown to induce IR mRNA levels in other cell types.[35]

It was not entirely unexpected to find that insulin promotes chondrogenesis in passaged cells as this effect has been reported in other cell types, such as MSCs and the ATDC5 cells (mouse teratocarcinoma cell line that differentiates to chondrocytes in the presence of insulin).[36,37] Proteomic studies suggest that insulin levels in FBS are much lower than that used in SF3D, which might explain why the presence of 20% FBS in SC3D was insufficient to promote matrix formation and accumulation by P2 cells.[12] The involvement of insulin was supported by the observation that treatment with HNMPA-(AM3) downregulated IR gene and protein levels, decreased sox9 and -6 levels, and decreased synthesis of collagens. However, HNMPA-(AM3) is known to have other effects that could affect chondrogenesis. It can block prostaglandin synthesis by chondrocytes,[38] which has been shown to affect chondrogenesis; hence, HNMPA-(AM3) could be working by mechanisms other than just modulating insulin effects.[39] In our culture system insulin may act via Shc as the levels of pShc and downstream ERK-MAPK were modulated by HNMPA-(AM3). In keeping with this observation microarray analysis during redifferentiation showed upregulation of Shc binding protein and PRKCSH (data not shown), both involved in Shc-mediated cascade.[40] Interestingly only the ERK-2 (42 kDa) and not the ERK-1 (44 kDa) subunit was affected by HMNPA-(AM3) treatment. Differential ERK-1/2 responses have been described by others as well.[41,42] Several studies have shown that under physiological conditions ERK-2 can exist in predominantly a monomeric form (not dimerized to ERK-1) and is capable of regulating downstream signaling pathways.[42] It was unexpected that ERK-2 levels would increase with IR inhibition by HNMPA-(AM3) treatment, but others have also observed that insulin can inhibit ERK, although in neuronal cells.[43] Further, our results are consistent with those that have shown that high levels of ERK-2 inhibit differentiation in mouse embryonic stem cells.[44]

The data also suggests that the level of Sox9 may be important for redifferentiation, which would be consistent with the study on liver cells where Sox9 was shown to affect differentiation in a dose-dependent manner.[45] In our system the redifferentiating cells have higher levels of Sox9 and subsequently higher association with *Col2a1* on chromatin when compared with cells treated with HMNPA-(AM3), a condition that prevented the increase in collagen synthesis. In addition, this treatment—although affecting levels of sox9 and sox6—did not seem to affect the formation of the transcriptional complex, at least as investigated in this study, as the cotranscriptional molecules sox6 and Med12 were detected. This suggests that insulin-induced increases in Sox9 levels may be involved in the redifferentiation and/or accumulation of matrix by passaged cells.

In summary we describe a novel serum- and TGFβ-free culture system that requires insulin, dexamethasone, and HG to generate hyaline-like cartilage tissue from passaged bovine chondrocytes grown in 3D on collagen-type-II-coated membrane inserts. This is an important step toward the ultimate goal of developing autologous, patient-specific tissue-engineered hyaline-like cartilage tissue suitable to use for repair/replacement of damaged articular cartilage. Further studies are required to translate what has been learnt from this SF3D study for use with human chondrocytes.

Acknowledgments

The authors thank Mr. Harry Bojarski and Ryding-Regency Meat Packers for providing bovine tissues. The project was supported by funding from U.S. Department of Defense. N.A. received a fellowship from the Samuel Lunenfeld Research Institute Special Opportunities Fund. J.I. was supported by OGSST, ON. C.E.B. was funded by BRAVO!/MHIRT and by NIH grant No. MD 001427.

Disclosure Statement

No competing financial interests exist.

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Received: September 10, 2013
Accepted: January 31, 2014
Online Publication Date: April 30, 2014