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High-Throughput Screening Assay for the Identification of Compounds Enhancing Collagenous Extracellular Matrix Production by ATDC5 Cells

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Extracellular matrix (ECM) provides not only structural support to the cells but also signals that regulate their fate. In many tissue engineering approaches, it remains challenging to achieve the right amount and type of ECM secreted by the cells to faithfully mimic the native tissue. In this article, we describe how to design and perform a high-throughput assay to screen for molecules capable of enhancing collagenous ECM (cECM) production. We chose ATDC5 cells to validate the assay since we want to use this chondrogenic cell line later for tissue engineering of hypertrophic cartilage. We used a fluorescently labeled collagen-binding probe to quantify total collagen content in ATDC5 cultures. The LOPAC¹²⁸⁰ library of pharmaceutically active compounds was screened using insulin (a known inducer of cECM in ATDC5 cells) as positive control. After screening and validation, the small-molecule tetradecylthioacetic acid (TTA) was shown to enhance cECM production by ATDC5 cells at both gene expression and protein level. Moreover, when combined with insulin, TTA showed a synergistic effect on cECM production. In contrast, exposure of human primary chondrocytes and human mesenchymal stromal cells to TTA did not induce cECM secretion. In conclusion, we have developed an HTS assay to screen for compounds capable of enhancing cECM production and discovered TTA as a potent enhancer of cECM secretion by ATDC5 cells.

Introduction

MANY DIFFERENT CELL types secrete an extracellular matrix (ECM), which provides mechanical support and serves as a signaling hub conveying signals from the cells to the surrounding environment and vice versa. Some of these signals control important biological functions such as cell viability and cell fate.^{1,2} In tissue engineering, a common procedure is to isolate cells from their native tissue while leaving behind the ECM. The cells are then expanded to increase numbers, and are reaggregated to mimic a three-dimensional (3D) environment, either by the use of a scaffold or simply by cell aggregation. Ideally, the cells in the tissue-engineered construct will adhere, proliferate, and secrete ECM. The nature of secreted ECM can be, to a certain extent, controlled by the signals provided by the scaffold (e.g., topographical cues) or by soluble signals to which the cells are exposed (e.g., growth factors or small molecules).^{3–5}

In bone tissue engineering through endochondral ossification, the amount of hypertrophic cartilage matrix in the tissue-engineered construct is crucial for successful bone formation. We reported previously a method to efficiently generate bone *in vivo* using mouse embryonic stem cells predifferentiated into hypertrophic chondrocytes *in vitro*.⁶ The amount of bone detected in the construct after implantation was highly correlated with the amount of hypertrophic cartilage observed *in vitro*. Interestingly, the capacity of hypertrophic cartilage matrix to induce bone formation *in vivo* is not only limited to living cartilage. In 1958, Bridge and Pritchard performed a series of experiments, in which devitalized tissues were placed under the kidney capsules or implanted subcutaneously in the ears of rabbits. Tissues containing hypertrophic cartilage, which were devitalized with alcohol, acetone, and HCl, or heated to 55°C consistently, formed bone.⁷ Later, Marshall Urist also observed new bone formation when a boiled fracture callus was transplanted into the anterior chamber of a rat's eye.^{8–10} This interesting phenomenon can

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be exploited to produce off-the-shelf bone-inducing hypertrophic cartilage since devitalized allografts provoke a much milder immune response compared with viable tissue.^{11,12} We attempt to repeat the devitalized cartilage experiment, but instead of using natural hypertrophic tissue, such as growth plate cartilage or fracture callus, we try to tissue engineer hypertrophic cartilage *in vitro* to better control the state of hypertrophy. However, one final hurdle remains: production of sufficient amounts of ECM *in vitro*.

Previously, we used high-throughput screening (HTS) to search for small molecules that can modulate the cellular response of certain cell types. For instance, using HTS, we identified several compounds that improve osteogenic differentiation of the human mesenchymal stromal cell (hMSC)¹³ and discovered that the small-molecule phenanthroline is very efficient in inducing angiogenesis.¹⁴

In this article, we designed and performed an HTS assay to search for compounds that can enhance ECM production. We chose the chondrogenic cell line, ATDC5, for this HTS assay because it is a well-established cell line able to produce collagenous ECM (cECM) while growing in two-dimensional (2D) culture on normal tissue culture-treated polystyrene surface, which simplifies the detection method. More importantly, ATDC5 cells can consistently differentiate into hypertrophic chondrocytes and produce hypertrophic cartilage matrix *in vitro*,¹⁵ which can be used later for our devitalized cartilage strategy. To quantify the amount of collagenous matrix produced by the cells, we use a fluorescently labeled collagen-binding probe, which has affinity for both fibrillar and nonfibrillar collagen.^{16,17} This property, that is, not specific to any particular type of collagen, was exploited to measure the total amount of cECM produced by the cells. Using this assay, we identified and validated tetradecylthioacetic acid (TTA) as a compound capable of enhancing cECM production by ATDC5 cells.

Materials and Methods

Cell culture

The ATDC5 cell line was obtained from the RIKEN cell bank (Japan) and maintained in basic medium (BM), which consists of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 supplied with GlutaMAX™ (DMEM/F-12 GlutaMAX), 5% v/v fetal bovine serum (FBS; Lonza), 0.2 mM L-ascorbic acid-2 phosphate (ASAP), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (PS). Cells were grown at 37°C in a humid atmosphere with 5% CO₂. The medium was refreshed twice a week and cells were used for further subculturing or cryopreservation upon reaching near confluence. To stimulate chondrogenesis and ECM production, BM was replaced with insulin medium (IM) consisting of BM supplemented with 1× insulin, transferrin, selenium solution (ITS) to yield a final concentration of 10 µg/mL insulin, 5.5 µg/mL transferrin, and 3.87 × 10⁻⁸ M sodium selenite.¹⁸

Primary human mesenchymal stem cells (hMSCs, three donors) and human primary articular chondrocytes (hPCs, three donors) were obtained and isolated as described previously.^{19,20} The use of bone marrow aspirates and human knee biopsies was approved by the Medical Ethics Committee of Medisch Spectrum Twente, and informed consent was obtained from all patients. hMSCs were expanded at the initial

seeding density of 1000 cells/cm² in proliferation medium consisting of α-MEM, 10% v/v FBS (Lonza), 2 mM L-glutamine, 0.2 mM ASAP, PS, and 1 ng/mL recombinant human basic fibroblast growth factor (AbD Serotec). For chondrogenic differentiation, hMSCs (passage 2) were seeded at 2.5 × 10⁵ cells per well in Cellstar U-shaped 96-well plates (Greiner Bio-one) and centrifuged for 3 min at 300 g to form pellets. Chondrogenic medium for hMSCs consists of DMEM high glucose, 100 µg/mL sodium pyruvate, 0.2 mM ASAP, PS, 1× ITS, 0.1 µM dexamethasone (Dex), and 10 ng/mL transforming growth factor 3 (TGFβ3; R&D systems, 243-B3-010). hMSC pellets were cultured for 28 days, and the medium was changed two to three times per week. hPCs were expanded in chondrocyte proliferation medium consisting of DMEM, 10% v/v FBS, 1× nonessential amino acid, 0.2 mM ASAP, 0.4 mM proline, and PS. For chondrogenic differentiation, hPCs (passage 1) were seeded at 10,000 cells/cm² in six-well plates. Chondrogenic medium for hPCs was the same as for hMSCs with the addition of 2 mM L-glutamine. hPCs were cultured for 14 days, and the medium was changed two to three times per week. All media and medium supplements were purchased from Life Technologies; all common chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

Collagen probe CN35-AF488 assay

Cells were cultured on a 96-well black/clear imaging plate (BD Bioscience; 353219). Per time point, the culture medium was replaced with staining medium, which consists of 0.25 mM CN35-AF488 diluted in DMEM/12 without supplements. CN35-AF488 consists of a collagen-binding protein domain (CNA35) conjugated to the fluorescent dye, Alexa Fluor 488.^{16,17} Using solid-phase binding assays and immunohistological staining, Krahn *et al.* showed that the collagen probe bound relatively well to all types of collagen that were tested with a different affinity for the different collagen types (I–VI), but did not bind to any of the other ECM proteins.¹⁶ The plates were incubated at 37°C in a humid atmosphere with 5% CO₂ on an orbital shaker (20 rpm) for 1 h. After that, the plates were washed thrice with PBS (Gibco) to remove the unbound probe. Then, total fluorescent intensity of CNA35-AF488 was measured at 520 nm on a Victor plate reader (PerkinElmer).

High-throughput screen

ATDC5 cells were seeded at 5000 cells/cm² in a 96-well black/clear imaging plate (BD Bioscience; 353219) in BM and allowed to proliferate for 3 days. Then, the 1280 test compounds (LOPAC1280; Sigma Aldrich) were added at a final concentration of 4.5 µM in BM. BM and IM were used as negative (NC) and positive (PC) control, respectively. DMSO, the solvent used in the library at 0.25% v/v, was added to the controls. The medium, including compounds, was changed after 3 days, and after 6 days, the collagen probe CNA35-AF488 assay was performed. A compound was defined as a hit if its signal was greater than the mean plus three times standard deviation of the negative controls in the same plate.

Hits validation

Three different concentrations of each hit compound were tested for their capacity to enhance ECM production. The

experimental protocol was the same as the primary screen, with the exception that each compound and concentration was tested in triplicate. A PrestoBlue assay was performed to assess the effect of the compound on metabolic activity, representing cell viability. Briefly, BM containing 10% (v/v) PrestoBlue solution (Invitrogen) was added and incubated at 37°C for 1 h. Then, fluorescence was measured at 590 nm on a Victor plate reader (PerkinElmer). The concentration that induced the highest secretion of cECM without affecting the metabolic activity was selected for subsequent experiments.

Gene expression analysis

ATDC5 cells (5000 cells/cm²) and hPCs (10,000 cells/cm²) were seeded in triplicate in six-well plates in BM and allowed to attach overnight. hMSCs (2.5×10^5 cells per well) were aggregated in U-shaped 96-well plates in hMSC chondrogenic differentiation medium and allowed to form pellets overnight. Then, the compounds were added, and the medium was changed two to three times per week. At selected time points, RNA was isolated using an RNA II nucleospin RNA isolation kit (Machery Nagel). For chondrogenic differentiation of hMSCs, three pellets were pooled for RNA isolation. RNA concentrations were measured using an ND100 spectrophotometer (Nanodrop1000). cDNA was synthesized from 1 µg of RNA using iScript (BioRad) according to the manufacturer's protocol. qPCR was performed using 50 ng of cDNA, 0.4 µM of each forward and reverse primer (Sigma Genosys), and 1x SensiMix SYBR and Fluorescein master mix (Bioline). Primer sequences are shown in Supplementary Table S2 (Supplementary Data are available online at www.liebertpub.com/tec). Real-time qPCR was performed in a Biorad My IQ5 machine (Biorad). Data were analyzed using the fit point method of My IQ5 software. The baseline was calculated automatically by the software at the lower log-linear part above baseline noise, and the crossing temperature (Ct value) was determined. Ct values were normalized to the Beta-2 microglobulin (B2M) housekeeping gene, and Δ Ct (Ct, control–Ct, sample) was used to calculate the upregulation in gene expression.

Total glycosaminoglycan quantification assay

Glycosaminoglycan (GAG) assay was performed as described previously.²⁰ Briefly, samples were washed with PBS and frozen at –80°C. Subsequently, they were digested with 1 mg/mL proteinase K (Sigma-Aldrich) in Tris/EDTA buffer (pH 7.6) containing 18.5 mg/mL iodoacetamide and 1 mg/mL pepstatin A (Sigma-Aldrich) overnight at 56°C. GAG content was spectrophotometrically determined with 9-dimethylmethylene blue chloride (DMMB; Sigma-Aldrich) staining in PBE buffer (14.2 g/L Na₂HPO₄ and 3.72 g/L Na₂EDTA at pH 6.5) with a microplate reader (Bio-TEK instruments) at 520 nm. The standard curve for the GAG analysis was generated using chondroitin sulfate A (from bovine trachea; Sigma-Aldrich).

Hydroxyproline assay

A hydroxyproline assay kit was purchased from Biovision (K555-100). The assay was performed according to the manufacturer's protocol. Briefly, samples (cell layer) in triplicate were retrieved from tissue culture flasks at weeks

1, 2, 3, and 4 using cell scrapers and transferred to pressure-tight Teflon-capped vials for hydrolysis in 6M HCl for 3 h at 120°C. Lysates were transferred to a 96-well plate and dried under vacuum. Chloramine T and 4-(Dimethylamino)benzaldehyde (DMAB) reagent were added to each sample and incubated for 90 min at 60°C. Absorbance at 560 nm was measured in a microplate reader (Bio-TEK instruments). The standard curve was generated using 4-hydroxyproline as indicated in the manufacturer's protocol.

Statistics

One-way or two-way ANOVA with Bonferroni's post-test and Pearson correlation analysis were performed using GraphPad Prism version 6.02 for Windows, GraphPad Software, www.graphpad.com.

Results

ECM production in differentiating ATDC5 cells

ATDC5 cells produce high amounts of cECM in 2D culture. To characterize our ATDC5 cell line, we cultured the cells for 3–4 weeks in BM and IM and analyzed their morphology and the expression profile of cECM-related genes. Figure 1A shows the morphological changes of the ATDC5 cell layer upon exposure to IM over 3 weeks. At day 7, the cell layer morphology was similar for both conditions. At day 14, the ATDC5 cell layer treated with insulin developed many aggregates, which stain positive for Alcian Blue (data not shown), and further developed into nodules after 21 days. In contrast, in BM, small aggregates appeared at day 21, 1 week later than in IM. However, when cultured for up to 40 days, these aggregates did not progress into nodules as seen in the IM (data not shown). The ATDC5 nodules did not stain positive for Alizarin Red (data not shown) unless the medium was switched to mineralization medium supplemented with calcium and phosphate salts. Gene expression analysis showed an increase in mRNA levels for collagen type II and type X in BM and IM over a 4-week time course (Fig. 1B). After 1 week, IM led to a 5-fold increase in collagen type II expression compared with BM, which increased to a 13-fold change upon 2 weeks and decreased thereafter to its basal level. A similar profile was observed for collagen type X expression (although in this case the fold change between the conditions reached a maximum of 60 at 2 weeks). A delay in the expression of both genes was observed in BM with maximum expression observed after 4 weeks instead of two for IM.

Quantification of total collagen

To quantify the total collagen content, we stained the cells with the CNA35-AF488 collagen probe. Figure 2A shows fluorescent images of ATDC5 cells, grown in BM and IM for 10 days, stained with the collagen probe. While ATDC5 cells also produce a basal level of collagen in the BM, the collagen network becomes much denser in the IM. The time point day 10 was chosen in our preliminary experiments; after this time, the cell layer in IM was already too thick and could detach easily in the staining and washing steps, affecting the fluorescent quantification. To demonstrate the capacity of the collagen probe assay, we compared it with a common GAG assay in a dose–response experiment of

FIG. 1. Characterization of ATDC5 cell line. (A) Representative microscopy pictures of ATDC5 cultured on treated polystyrene surface (TCP) at days (D) 7, 14, and 21 in basic medium (BM) (top panel) or insulin medium (IM) (bottom panel). Scale bar represents 1 mm. (B) Gene expression profile of collagen type II and X in ATDC5 cultured on TCP in BM (blue line) or IM (red line) at D0, weeks (W) 1, 2, 3, and 4. ($n=3$). Color images available online at www.liebertpub.com/tec

FIG. 2. Sensitivity of collagen probe CNA35-Alexa488 to detect extracellular matrix (ECM). (A) Fluorescence images of ATDC5 cultured on TCP for 10 days in BM or IM, stained with collagen probe CNA35-Alexa488, and visualized using a BD pathway 432 microscope. Scale bar represents 100 μm . Dose response of ATDC5 to insulin after 10 days cultured on TCP, (B) stained with collagen probe CNA35-Alexa488, and total fluorescence intensity measured by a Victor plate reader, or (C) quantified by GAG assay. Error bars represent standard deviation ($n=3$). Statistical analysis was performed using one-way ANOVA and Bonferroni's post-test. (*) denotes $p<0.05$ compared with basic (0 $\mu\text{g/mL}$ insulin) and (***) denotes $p<0.001$ compared with basic. Color images available online at www.liebertpub.com/tec

insulin on ATDC5 cells. Total fluorescence intensity of the CNA35-AF488 using the collagen probe assay (Fig. 2B) was highly correlated with increasing insulin concentration ($R^2=0.8$, $p=0.0066$). Using the GAG assay (Fig. 2C), total GAG content and insulin concentration were less correlated ($R^2=0.37$, $p=0.1442$). Thus, a collagen probe assay has better resolution in measuring the response of ATDC5 cells to changes in stimuli.

Screening for compounds enhancing ECM production by ATDC5 cells

To find small molecules capable of enhancing cECM production by ATDC5 cells, we screened the LOPAC1280 library (containing 1280 different compounds) using total fluorescence intensity of the collagen probe CNA35-AF488 staining as readout (Supplementary Fig. S1A). Supplementary Figure S1B shows the mean and standard deviation of the PC and NC

in every plate. Total cECM content was significantly increased ($p<0.0001$) when insulin was added (1.5-fold increase in total fluorescence intensity: NC = 15264 ± 1552 vs. PC = $22,718 \pm 1294$). Since insulin is a very potent cECM inducer for ATDC5 cells, no compound was able to induce cECM secretion to a higher level than that of the IM in this screen. Thus, we decided to define hits based on their ability to enhance cECM production to a significantly higher level than BM, for which a hit selection threshold was chosen as the mean plus three times the standard deviation of the negative controls in the same plate. Based on this criterion, we identified in total 13 hit compounds (Supplementary Table S1), 6 of which were ordered for further testing. Selected hits were further tested using a wider range of concentrations (2–200 μM) to confirm the cECM-inducing effect on ATDC5 cells. As in the HTS assay, BM and IM were used as NC and PC, respectively. Metabolic activity assay was performed to examine potential cytotoxic effects of the compounds. Of the six compounds tested, only DL-Cycloserine

FIG. 3. Hit identification and validation. Representative figures show how hits were identified and validated for (A)-plate 1 (identify taucholic acid), (B)-plate 4 (identify DL-Cycloserine), and (C)-plate 15 (identify tetradecylthioacetic acid). Plate number is specified in the LOPAC¹²⁸⁰ catalog. Heat maps (left) show relative total fluorescence intensity of each well on the plate compared with the PC and NC. Bar graphs (middle) show the effects of different concentrations of hit compounds on metabolic activity of ATDC5. Bar graphs (right) validate the capability of hit compounds in different contractions to enhance collagenous ECM production of ATDC5, quantified by the collagen probe assay. Error bars represent standard deviation ($n=3$). Statistical analysis was performed using one-way ANOVA and Bonferroni's post-test. (*) denotes $p<0.05$ compared with NC, (**) denotes $p<0.01$ compared with NC, and (***) denotes $p<0.001$ compared with NC. Color images available online at www.liebertpub.com/tec

and tetradecylthioacetic acid (TTA) were able to reproduce the cECM-inducing effect found on the HTS assay, inducing a significantly higher amount of cECM compared with the NC (Fig. 3B, C).

Expression of ECM genes

Next, we investigated the effect of the selected compounds on ATDC5 cells at the mRNA level. There are many

different cECM proteins, but we were mostly interested in collagen type II and aggrecan since these cECM proteins form the major structural component of cartilage, especially articular cartilage. ATDC5 cells were cultured in the presence of the selected compounds for 10 days. The concentrations tested were the ones that induced the highest amount of cECM in the previous validation experiment. IM was again used as a positive control and compared with BM, it upregulated collagen type II expression 2.5 times and

FIG. 4. Further validation of hit compounds by gene expression analysis. Collagen type II expression (A) and aggrecan expression (B) of ATDC5 treated with the hit compounds for 10 days. The concentration of the compounds was determined from the previous validation experiments. Only tetradecylthioacetic acid (TTA) showed a significant difference compared with the NC and thus was further tested in a dose-response experiment. (C–E) Dose-response effect of TTA on ATDC5. TTA was added at different concentrations to BM and IM. Collagen II expression (C), aggrecan expression (D), and total collagen quantification by collagen probe assay (E) were measured after 10 days. Notably, when combined with IM, TTA exerted a synergistic effect on aggrecan expression of ATDC5. Error bars represent standard deviation ($n=3$). Statistical analysis was performed using one-way ANOVA and Bonferroni's post-test. (*) denotes a significant difference compared with the BM and (#) denotes a significant difference compared with the IM. (*) or (#) denotes $p < 0.05$, (**) or (##) denotes $p < 0.01$, (***) denotes $p < 0.001$, and (****) or (####) denotes $p < 0.0001$.###, ns, non-significant.

aggrecan 9 times. From the tested compounds, only 20 μ M TTA was able to induce a significantly higher expression of collagen type II (2-fold) and aggrecan (2.6-fold) compared with the BM (Fig. 4A, B). Based on this result and the results from the previous experiment, we decided to select TTA for further testing.

To further explore the effect of TTA on ATDC5 cells, we tested TTA in a narrower range of concentrations spanning the 20 μ M previously tested (from 10 μ M to 80 μ M). Furthermore, we exposed cells to both TTA and insulin to see whether they could have a synergistic or additive effect on cECM production by ATDC5 cells. Interestingly, the expression of collagen type II (Fig. 4C) and aggrecan (Fig. 4D) was significantly higher when TTA was added to IM. At the protein level (using the collagen probe assay), 10 μ M TTA combined with IM induced a significantly higher amount of ECM compared with IM alone (Fig. 4E).

Effect of TTA in long-term culture with ATDC5

To test the effect of TTA on ATDC5 cells in long-term culture, we cultured ATDC5 cells in BM and IM with or without 20 μ M TTA for 4 weeks. Figure 5 shows representative pictures of the cell layers in different conditions and different time points. Over time, the cell layers in all conditions developed cell aggregations, which grew into nodules at later time points. In the presence of 20 μ M TTA, this process was accelerated. Moreover, with TTA, the nodules were often bigger and denser. Gene expression analysis was performed for collagen type II, collagen type X, aggrecan, and *SOX9* (Fig. 6A–D). In the presence of 20 μ M TTA, expression of all these genes was upregulated compared with the conditions without TTA (except for *SOX9* at week 3 and 4). The upregulation was more pronounced between weeks 2 and 4.

A hydroxyproline assay was also performed to estimate total collagen content of the ECM derived from ATDC5 cells (Fig. 6E). Although hydroxyproline content of all conditions increased consistently over time, we did not find any statistically significant difference between the TTA conditions and other conditions mostly due to the large technical variation in the different replicates. Even the difference in the amount of hydroxyproline in IM versus BM was not evident until week 4 when a statistically significant difference was observed.

Effect of TTA on other cell types

We confirmed that TTA was capable of enhancing cECM production and inducing collagen type II and aggrecan expression in ADTC5 cells. Thus, we decided to test the effect of TTA on other cell types to see whether its effect is cell-type dependent. For this purpose, we chose human primary articular chondrocytes (hPCs) and hMSCs, the two most clinically relevant cell types for cell-based therapies aimed at cartilage repair. Three different donors were tested for each cell type to correct for donor variability. Basic and chondrogenic media were used as negative and positive controls, respectively. The concentration of TTA used was 10 μ M, determined by preliminary experiments using metabolic activity assay to exclude toxic concentrations (data not shown). Since hPCs are known to produce cECM in culture, we grew them on tissue culture plastic for 14 days, and then performed gene expression analysis for collagen type II, aggrecan, and *SOX9*. hMSCs were chondrogenically differentiated in pellet cultures for 30 days, followed by gene expression analysis also on the same set of genes.

For hPCs, 10 μ M TTA combined with chondrogenic medium increased the expression of aggrecan in all three donors (statistically significant in two out of three donors).

FIG. 5. ATDC5 cell layer in long-term culture with TTA. Representative microcopy pictures of ATDC5 cultured on TCP at weeks 1–4 in BM, BM+20 μ M TTA, IM, and IM+20 μ M TTA. Scale bar represents 1 mm. Color images available online at www.liebertpub.com/tec

FIG. 6. The effect of TTA in long-term culture with ATDC5. Gene expression profile of collagen type II (A), collagen type X (B), aggrecan (C), and *SOX9* (D), plus hydroxypoline content (E) of ATDC5 cultured on TCP in BM (solid blue line), BM supplement with 20 μM TTA (broken blue line), IM (solid red line), and IM supplemented with 20 μM TTA (broken red line) at weeks 1, 2, 3, and 4. Error bars represent standard deviation ($n=3$). Statistical analysis was performed using two-way ANOVA and Bonferroni's post-test. (*) denotes a significant difference compared with the BM at the same time point and (#) denotes a significant difference compared with the IM at the same time point. (*) or (#) denotes $p < 0.05$, (**) or (##) denotes $p < 0.01$, (***) or (###) denotes $p < 0.001$, and (****) or (####) denotes $p < 0.0001$. Color images available online at www.liebertpub.com/tec

However, either used alone or in combination with chondrogenic medium, TTA downregulated the expression of collagen type II and *SOX9* in this cell type (Fig. 7A). Interestingly, the same trend was seen in hMSCs when 10 μM TTA significantly downregulated collagen type II and *SOX9* expression in two of three donors (Fig. 7B). In the other donor (D301), collagen type II and *SOX9* expression was upregulated (not significantly), and aggrecan expression increased 30-fold compared with the negative control when TTA was added. However, when combined with chondrogenic medium, TTA always reduced expression of these genes compared with the chondrogenic medium condition.

Discussion

Designing and performing an HTS requires that a number of issues are addressed. In this screen, we chose insulin as a positive control because insulin is widely used to induce ECM secretion of ATDC5 cells. +Using total fluorescent intensity of the collagen probe as a readout, even insulin was only able to induce a 1.5-fold, in total, fluorescence intensity. Still, of the six hits selected for validation from the recent screen, DL-Cycloserine and TTA could reproduce the positive response observed with insulin, although to a lesser extent. In later validation experiments, using a more sensitive technique such as qPCR with higher concentration and longer

exposure time, we were able to redeem TTA as a true enhancer of cECM expression for ATDC5 cells. This could not have been done in the actual screen since all compounds were only tested at one concentration and one exposure time. Our screen was designed to be exploratory because a universal mechanism for inducing cECM protein production is not known. For this purpose, we chose a nonspecific readout: total amount of collagen estimated by total fluorescence intensity from a collagen probe staining, which is not specific for any type of collagen. The most advantageous point of the collagen probe staining is its ease of use and thus is ideal for an HTS. The three step protocol, adding the dye, washing, and quantifying, can be performed in any laboratory manually or automatically. Its main problem is the instability of the fluorophore; even though Alexa Fluor is very good, photo bleaching will always occur. We addressed this problem by performing the staining and quantification in batches and always included both the positive and negative controls on every plate. Since the collagen probe recognizes the amino acid sequence of collagens,^{16,21} it will also bind to them in solution. However, the complex remains in solution and will be removed in the washing step. The collagen probe can also bind to collagen gel (with chopped fiber) and gelatin (denatured collagen), thus it is not suitable to use in a gel system.

Other methods that can be used to quantify collagen include hydroxypoline assay and Picro Sirius red assay.

FIG. 7. Effect of TTA on human primary chondrocytes and human mesenchymal stromal cells. (A) hPCs (three donors) were cultured on TCP in chondrocyte basic medium (CBM), CBM+10 μ M TTA, chondrogenic differentiation medium (CDM), and CDM+10 μ M TTA. Gene expression analysis on collagen type II, aggrecan, and *SOX9* was performed after 14 days in culture. (B) hMSCs (three donors) were cultured in pellets of 2.5×10^5 cells in hMSC basic medium (MBM), MBM+10 μ M TTA, hMSC chondrogenic differentiation medium (MCM), and MCM+10 μ M TTA. Gene expression analysis on collagen type II, aggrecan, and *SOX9* was performed after 30 days in culture. Error bars represent standard deviation ($n=3$ for each donor). Statistical analysis was performed using one-way ANOVA and Bonferroni's post-test for each donor. (**) denotes $p < 0.01$, (***) denotes $p < 0.001$, and (****) denotes $p < 0.0001$, ns, nonsignificant.

While the hydroxyproline is by far the most common method for quantifying tissue fibrosis and collagen deposition,²² its laborious protocol hinders its high-throughput capability. The hydrolysis step, which is necessary for the release of amino acid hydroxyproline from the protein collagen, requires high temperature and high pressure and thus is not suitable for normal plastic well plates. With some modifications, the Picro Sirius red staining can be used in an HTS very similar to our collagen probe assay.²³ When bound to collagen, the Sirius red–collagen complex will precipitate, making it suitable for quantifying even soluble collagens.

While we confirmed that TTA is capable of enhancing cECM production by ATDC5 cells, we did not attempt to study the mechanism behind it. To date, TTA is a well-known synthetic fatty acid and is the focus of many studies for its effect on fat metabolism-related disorders,²⁴ inflammation,²⁵ and even cardiovascular function.^{26,27} A lot of evidence points at the involvement of TTA in the peroxisome proliferator-activated receptor (PPAR)-mediated signaling.^{28–30} According to Forman *et al.*,³⁰ TTA belongs to a group of dual-function PPAR α activators, which also includes tetradecylthiopropionic acid (TTP), tetradecylglycidic acid (TDGA), octylthiopropionic acid (OTP), nonylthioacetic acid (NTA), LY 171883, and 2-bromopalmitate (2Br-C16).

All these compounds activate PPAR α directly as ligands; plus, as metabolic inhibitors of the mitochondrial β -oxidation pathway, they cause accumulation of endogenous fatty acids, which are also PPAR α ligands.

Some *in vitro* researches have shown the antiproliferative effect of TTA on certain cell types,^{31–33} which was not confirmed by our data. Exposure of ATDC5 cells, hMSCs, and hPCs to TTA did not have a noticeable negative effect on proliferation unless very high concentrations of TTA (200 μ M for ATDC5, 40 μ M for hMSCs and hPCs) were used, which led to a reduction in metabolic activity, followed by cell death (Fig. 3C, middle graph).

Since ATDC5 is a chondrogenic cell line, we wanted to see the same cECM-enhancing effect of TTA on hPCs, but unexpectedly, in our experimental conditions, TTA consistently inhibited the expression of collagen II in hPCs. This was also observed for hMSCs undergoing chondrogenic differentiation. We have yet to explain this phenomenon, but the fact that ATDC5 cells can produce abundant cECM over time even in BM, suggests that the mechanism of action of TTA on ATDC5 cells is different from that on hMSCs and hPCs. Westergaard *et al.* showed that TTA activated all human PPAR subtypes in the ranking order PPAR δ >> PPAR α > PPAR γ , whereas in the experiment using mouse PPARs,

TTA most efficaciously activated PPAR α .³³ Although this could not explain the contradictory effect that we have seen in our experiments, it showed that the mechanism of action of TTA may be different between species.

With the ECM-enhancing effect of TTA on ATDC5 cells confirmed, especially when used in combination with insulin, we obtained a new method to grow and upscale our ATDC5 cartilage model *in vitro*. With our initial plan of studying the endochondral ossification process using live or devitalized hypertrophic cartilage, we can now use TTA in combination with insulin to enhance the amount of tissue mass produced by ATDC5 for *in vivo* implantation without the need of scaffold material. While increasing collagen quantity is advantageous for our purpose, collagen structure and organization can greatly affect property and functionality of the ECM. In previous publications,^{16,17} we have shown the capability of the collagen probe in high-resolution imaging. Collagen organization can be visualized with the collagen probe even better than with second harmonic generation, which is currently the most effective method for viewing 3D collagen organization in tissues. However, this resolution is not high enough to visualize collagen fibril periodicity. In this article, we have yet to look at collagen structure and organization. In the near future, we will focus on using micropatterned surfaces to modify the collagen fibril organization of the ATDC5 cell layer.

In conclusion, we have developed an HTS assay using the collagen probe CNA35-AF488 to screen for compounds capable of enhancing cECM production by the cells, and as an example, we discovered TTA as a potent cECM enhancer of ATDC5. With the method we present, any laboratory can customize the screen by choosing a different target cell type (e.g., primary chondrocytes, hMSCs) and a different compound library; the result will be different compounds that respond well to their cell type in terms of collagen production.

Disclosure Statement

No competing financial interests exist.

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