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A-674563 increases chondrocyte marker expression in cultured chondrocytes by inhibiting Sox9 degradation

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ABSTRACT

The implantation of autologous chondrocytes is a therapeutic treatment for articular cartilage damage. However, the benefits are limited due to the expansion of chondrocytes in monolayer culture, which causes loss of chondrocytic characters. Therefore, culture conditions that enhance chondrocytic characters are needed. We screened 5822 compounds and found that A-674563 enhanced the transcription of several chondrocyte marker genes, including *Col2a1*, *Acan* and *Col11a2*, in mouse primary chondrocytes. Experiments using cycloheximide, MG132 and bafilomycin A1 have revealed that Sox9 is degraded through the ubiquitin-proteasome pathway and that A-674563 inhibits this degradation, resulting in larger amount of Sox9 protein. RNA sequencing transcriptome analysis showed that A-674563 increases the expression of the gene that encodes ubiquitin-specific peptidase 29, which is known to induce the deubiquitination of proteins. Although the precise mechanism remains to be determined, our findings indicated that A-674563 could contribute to culture conditions that expand chondrocytes without losing chondrocytic characters.

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1. Introduction

Articular cartilage covers the ends of bone and is responsible for the smooth motion of diarthrodial joints. Cartilage is composed of chondrocytes embedded in cartilage extracellular matrix. Cartilage extracellular matrix is produced by chondrocytes and consists of a collagen fibril network that provides scaffold for proteoglycans. Cartilage collagen fibrils are heterotypic fibrils composed of types II, IX and XI collagen molecules. Cartilage proteoglycans consist of glycosaminoglycans covalently attached to the protein aggrecan. Cartilage extracellular matrix provides articular cartilage with mechanical properties that confer smooth joint motion. Articular

Abbreviations: ACI, autologous chondrocyte implantation; iChon cells, induced chondrogenic cells; *Col2a1*, type II collagen $\alpha 1$ chain gene; *Acan*, aggrecan gene; *Col9a1*, type IX collagen $\alpha 1$ chain gene; *Col9a2*, type IX collagen $\alpha 2$ chain gene; *Col9a3*, type IX collagen $\alpha 3$ chain gene; *Col11a1*, type XI collagen $\alpha 1$ chain gene; *Col11a2*, type XI collagen $\alpha 2$ chain gene; *Col1a1*, type I collagen $\alpha 1$ chain gene; *Usp29*, ubiquitin-specific peptidase 29.

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cartilage damage seldom heals [1] and often leads to the degeneration and erosion of a broader area of cartilage, resulting in debilitating conditions such as osteoarthritis. Autologous chondrocytes implantation (ACI) has been employed to treat local defects of articular cartilage caused by trauma [2,3]. ACI consists of taking biopsies from less-weight bearing areas of articular cartilage, releasing chondrocytes from the cartilage pieces by digesting the cartilage extracellular matrix with collagenase, expanding the chondrocyte number in monolayer culture, and implanting the expanded chondrocytes into the focal defects. The expansion of chondrocytes in monolayer culture for ACI is inevitable, because the number of chondrocytes obtained from the biopsy is insufficient to repair the defect. However, removal of the extracellular matrix and the cultural expansion cause the chondrocytes to express fibroblastic character, preventing them from producing cartilage extracellular matrix [4–7], a phenomenon known as chondrocyte dedifferentiation. Therefore, although ACI has shown good clinical results, the repaired tissue produced by dedifferentiated chondrocytes consists of fibrocartilage and not hyaline cartilage, resulting in inferior performance.

For ACI to generate hyaline cartilage, a monolayer expansion culture that prevents chondrocyte dedifferentiation is desired. In

this study we searched for bioactive compounds which, when added to the medium, could induce chondrocytic characters in monolayer expansion culture.

2. Materials and methods

2.1. High-throughput screening using 290-2-14 iChon cells

A total of 5×10^5 290-2-14 induced chondrogenic cells (iChon cells) [8] were plated in a 10-cm dish and cultured in DMEM supplemented with 10% FBS in the absence of doxycycline at 37 °C for 5 days to induce dedifferentiation. The cells were trypsinized, and 6.5×10^3 cells were replated into each well of 96-well plates and cultured in DMEM supplemented with 10% FBS in the presence of 0.01 µg/ml doxycycline at 37 °C. 1 µM of one compound from 8 libraries (Table 1), which in total included 5822 compounds, was added to each well at the time of the replating. We changed the medium containing the compound 5 days later, continued the culture for another 5 days, and subjected the cells to GFP fluorescence using ArrayScan™ High-Content Systems (Thermo).

2.2. Preparation of primary chondrocytes from mice

Primary chondrocytes were isolated as described previously [9]. The epiphyseal cartilage of the humerus and femur were dissected from 5-day-old mice in PBS. Adherent soft tissues were physically removed from the cartilage after digestion with 3 mg/ml collagenase D (Roche) in DMEM at 37 °C for 90 min. Chondrocytes were released from the cartilage by soaking the tissue in 0.5 mg/ml collagenase D overnight. The released cells were collected by centrifugation ($400 \times g$ for 10 min at 20 °C) and resuspended in fresh medium. Cells were seeded into 10-cm plates at a density of 5×10^5 cells/well, cultured in DMEM supplemented with 10% FBS for 7 days, and frozen as stock. Stocked cells were thawed and seeded into 10-cm plates at a density of 1.5×10^5 cells/well and were cultured in DMEM supplemented with 10% FBS in the absence or presence of various concentrations of A-674563.

2.3. Real-time RT-PCR analysis

RNA was extracted from the 290-2-14 iChon cells cultured in the presence of compounds for 10 days by using RNeasy Mini Kits (Qiagen). RNA was extracted from primary chondrocytes cultured in the presence of vehicle (DMSO) or various concentrations of A-674563 for 3 days. The total RNA was digested with DNase to eliminate any contaminating genomic DNA. For real-time quantitative RT-PCR, 100 ng of total RNA was reverse-transcribed into first-strand cDNA by using ReverTra Ace (Toyobo) and random primers. The PCR amplification was performed in a reaction volume of 10 µl containing 4 µl of cDNA, 6 µl of SYBR FAST qPCR Master Mix (Kapa Biosystems) and 7900HT (Applied Biosystems). The RNA

expression levels were normalized to the level of cyclophilin B gene (*Ppib*) expression. The primers used are listed in Table 2.

2.4. Preparation of reagents

A-674563 (Adooq Bioscience, A11034) was dissolved in DMSO at concentrations of 100, 3 and 1 mM to prepare stock solutions. Cycloheximide (Sigma) and bafilomycin A1 (Sigma) were dissolved in DMSO at a concentration of 100 mg/ml and 100 µM, respectively, to prepare stock solutions. 10 mM ready-to-use MG132 solution (Sigma) was purchased. The stock solutions were diluted with culture medium for *in vitro* experiments.

2.5. Cell counts

Primary chondrocytes were cultured in the presence of vehicle or various concentrations of A674563 for 3 days. Then, the cells were trypsinized, and the number of living cells were counted using Countess (Thermo).

2.6. Immunoblotting

Primary chondrocytes were lysed in RIPA buffer (10 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM EDTA, 1% NP-40, complete protease inhibitors from Roche and phosphatase inhibitor cocktail 1 and 2 from Sigma-Aldrich) [10] and subjected to SDS page. The separated proteins were then electroblotted and immunostained with rabbit anti-Sox9 antibody (1:2000, Cell Signaling, 82630), rabbit anti-β-actin antibody (1:10000, Cell Signaling, #4967), rabbit anti-phospho-Akt antibody (1:2000, Cell Signaling, #4060), and rabbit anti-Akt antibody (1:2000, Cell Signaling, #4685). The immunostained membranes were washed three times for 10 min and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit antibodies for 1 h. The SuperSignal West Dura Extendable Duration Substrate (Thermo) and LAS4000 (GE Healthcare) were used for chemiluminescent immunodetection.

2.7. siRNA transfection

siRNAs (*Akt1-a*; *Akt1-b*, cat. no.1062312-454, -537) corresponding to mouse *Akt1* mRNA were purchased from Thermo. Stealth RNAi (High GC, 452000, Medium GC, 452001 and Low GC, 452002, Invitrogen) were used as negative controls. Primary chondrocytes were seeded in 10 cm plates at a density of 5×10^5 cells/well, cultured in DMEM supplemented with 10% FBS and transfected with *Akt1-a* or *Akt1-b* siRNA at the final

Table 1
Libraries used for the compound screenings.

Supplier	Conc	Unit	Sample number
ENZO-FDA	10	mM	636
ENZO-ICCB	1	mM	477
Microsource US drug/International drug	10	mM	1258
Kinase I (EMD/ENZO)	10	mM	320
Phizer/Selleck kinase/Myria	10	mM	295
Tocris	10	mM	640
SIGMA_LOPAC	10	mM	1280
Tocris/Selleck/Sigma	10	mM	916
			5822

Table 2
Sequences of the primers used for real-time RT-PCR.

Primer	Sequence (5' to 3')
Aggrecan S	GCCCTTCACGTGTAAAAAGG
Aggrecan AS	CAGGGAGCTGATCTCGTAGC
Col2a1 S	ACCCCCAGGTGCTAATGG
Col2a1 AS	GAACACCTTTGGGACCATCTT
Col9a1 S	CAATCCTCAGGTTTCTGTCTCT
Col9a1 AS	TCTCAGGGGGTGCACATGA
Col11a2 S	CCTGGACCCCTTGGAAG
Col11a2 AS	TCCCCCTTAGTCCCTTCT
Sox9 S	TATCTTCAAGGCGCTGCAA
Sox9 AS	TCGGTTTGGGAGTGGTG
Usp29 S	CAGCCAGAGCTCTACAGGAAA
Usp29 AS	TCAGCCAAGTGTGACGATGT
Cyclophilin b S	TTCTTCATAACCACAGTCAAGACC
Cyclophilin b AS	ACCTTCCTGACCATCCAT

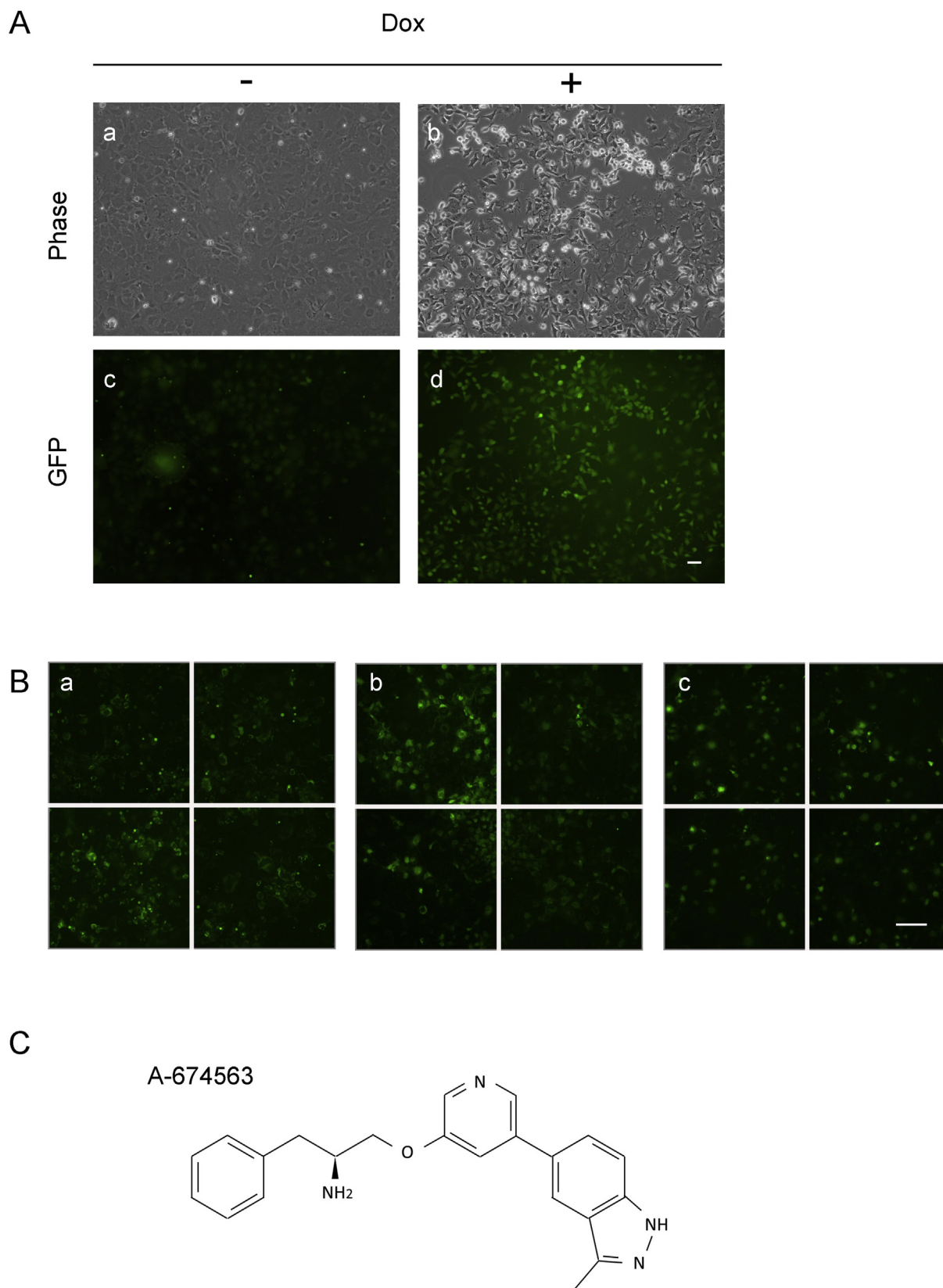


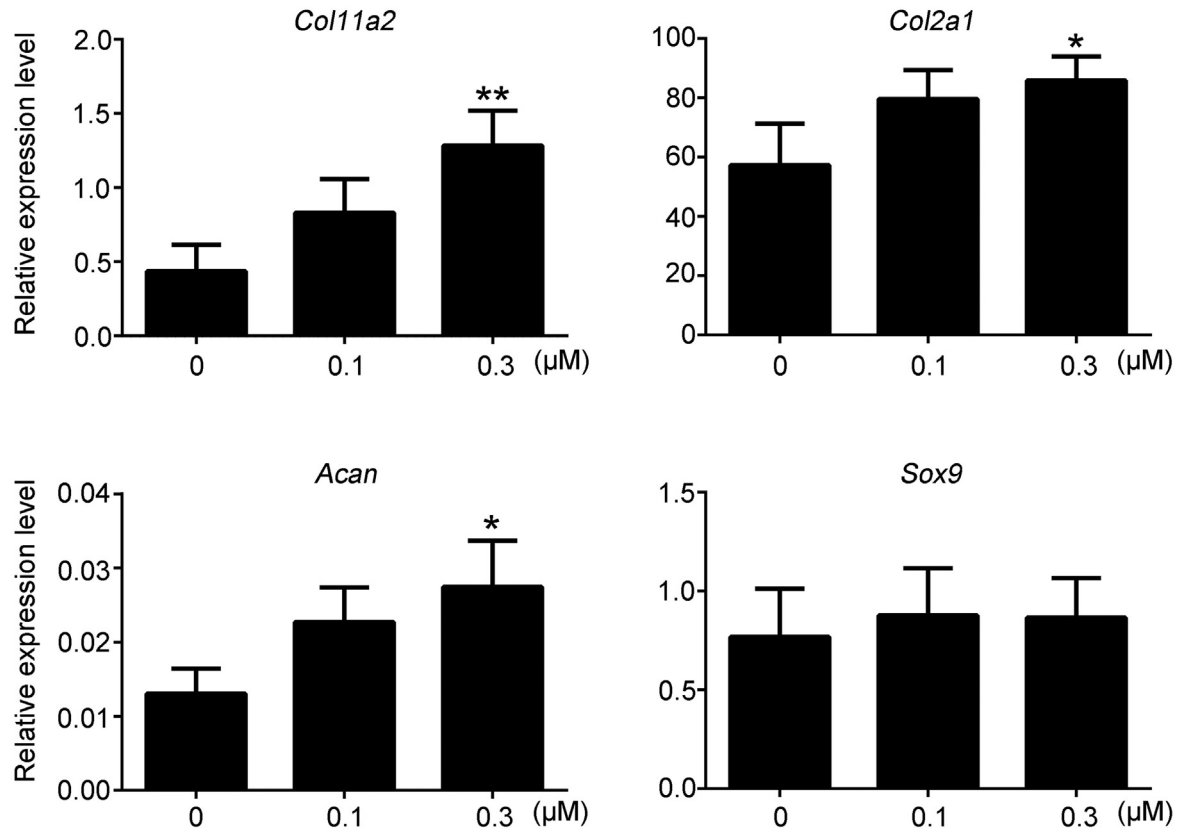
Fig. 1. High-throughput screening using 290-2-14 iChon cells.

(A) Images of 290-2-14 iChon cells cultured in the absence (a, c) or presence (b, d) of 1 $\mu\text{g/ml}$ doxycycline. Phase (a, b) and GFP (c, d) images. Bar, 100 μm .

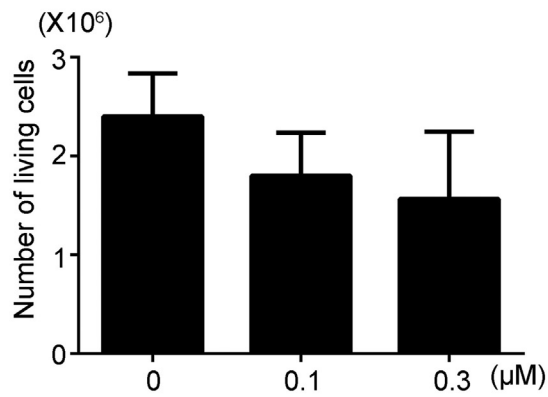
(B) GFP images of cells cultured in the presence of compounds (a, GALLAMINE TRIETHIODIDE, b, NSC 687852 and c, A-674563) for 10 days. Culture was done in the presence of 0.01 $\mu\text{g/ml}$ doxycycline. Each panel consists of images of four areas. Bar, 200 μm .

(C) Structure of A-674563.

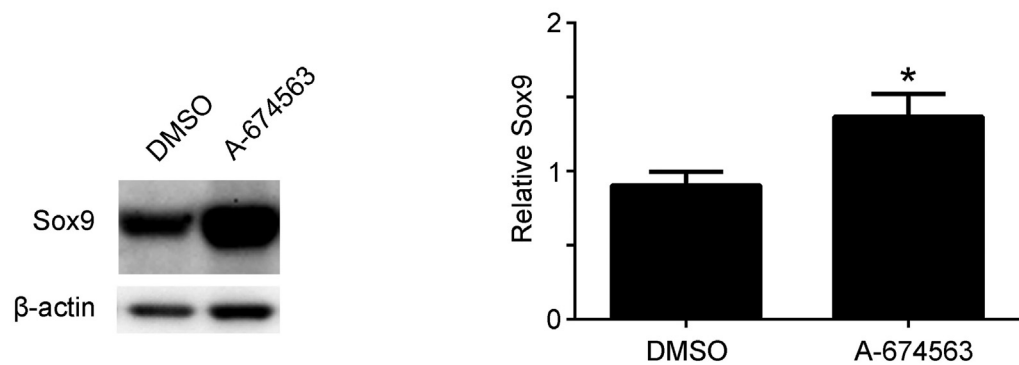
A



B



C



concentration of 25 nM using lipofectamine 2000 (Thermo). Three days later, cells were subjected to immunoblot analysis.

2.8. RNA sequencing analysis

RNAs were extracted from primary chondrocytes that had been cultured in the presence of vehicle or 0.3 μ M A674563 for 3 days using QIAzol Lysis Reagent (Qiagen) and miRNeasy Mini Kit (Qiagen). The quality of the extracted RNAs was evaluated by Bioanalyzer 2100 (Agilent Technologies). 1 μ g of total RNA was subjected to library preparation using TruSeq Stranded mRNA Library Prep Kit (Illumina) according to the manufacturer's instruction. The quality and quantity of the constructed libraries were evaluated by Bioanalyzer 2100 and Qubit dsDNA HS assay kit (Life Technologies). The libraries were sequenced in 75 cycles Single-Read mode of NextSeq 500 (Illumina). All sequence reads were extracted in FASTQ format using BCL2FASTQ Conversion Software (v2.17.1.14.). The adaptors, the poly-A sequences and the low-quality bases at the 3' read ends were trimmed by cutadapt-1.12. Untrimmed and trimmed reads were mapped onto the mouse genome mm10 using TopHat-2.1.1. We utilized mouse gene annotation from GENCODE release vM12. For gene expression analysis, the expression level of each gene was normalized to reads per kb of exon per million sequence reads (RPKM) using Cufflinks-2.2.1.

2.9. Statistical analysis

The data are shown as averages and standard deviations. The methods of statistical analysis are described in the figure legends. P values < 0.05 were considered to be statistically significant.

All experiments were approved by our institutional animal committee and institutional biosafety committee.

3. Results

3.1. High throughput screening for chemical compounds that induce Col11a2-EGFP expression

We previously showed that the transduction of c-Myc, Klf4 and Sox9 in dermal fibroblasts causes their conversion into chondrogenic cells through directed cell reprogramming [8,11]. We then established the 290-2-14 induced chondrogenic (iChon) cell line, which bears a *Col11a2-EGFP* reporter construct and doxycycline-inducible expression vectors of c-Myc, Klf4 and Sox9 [8]. The *Col11a2* gene encodes type XI collagen α 2 chains, which consist of cartilage extracellular matrix, and the *Col11a2-EGFP* reporter construct directs EGFP expression specifically in chondrocytes under the control of *Col11a2* promoter/enhancer sequences. We found that 290-2-14 iChon cells were flat and fibroblast-like and expressed little EGFP in the absence of doxycycline (Fig. 1Aa,c), which is consistent with dedifferentiated chondrocytes. The addition of 1 mg/ml doxycycline to these cells induced the expression of c-Myc, Klf4 and Sox9, a transformation to a polygonal or round morphology and the expression of EGFP, indicating directed cell reprogramming to chondrogenic cells (Fig. 1Ab,d).

We screened 5822 compounds on the 290-2-14 iChon cells.

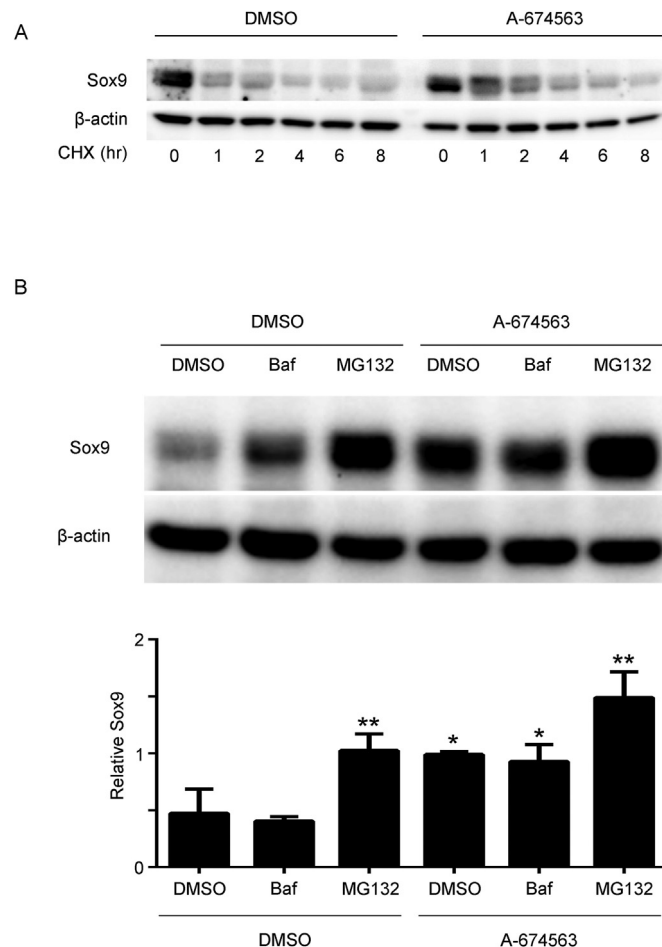


Fig. 3. Analysis of Sox9 degradation by A-674563 in primary chondrocytes. (A) Primary chondrocytes were cultured in the presence or absence of 0.3 μ M A-674563 for 2 days and treated with 100 μ g/ml cycloheximide. Cells were lysed after treatment for 0, 1, 2, 4, 6, and 8 h, and cell lysates were subjected to western blot analysis for Sox9 and β -actin. (B) Primary chondrocytes were incubated with 1 μ M MG132, 10 nM bafilomycin A1, or vehicle (DMSO) in the presence or absence of 0.3 μ M A-674563 for 5 h. *Top*, cell lysates were subjected to immunoblot analysis for Sox9 and β -actin. *Bottom*, signal intensities were analyzed with Image Quant TL (GE). Data are shown as means $\pm$ s.d. *P < 0.05 and **P < 0.01 by two way ANOVA followed by the Tukey post-hoc test (n = 3 wells).

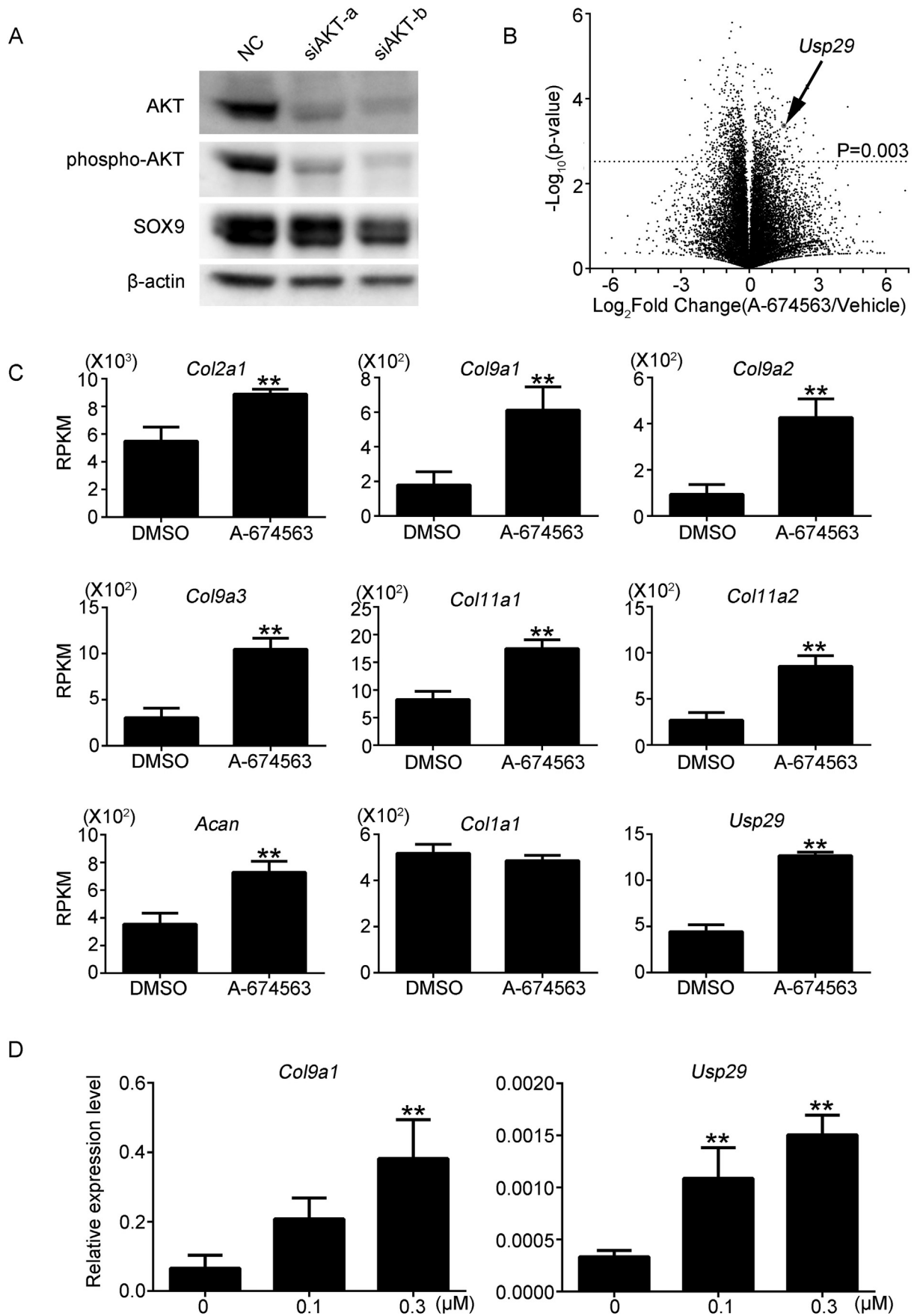
Before the start of the screening, the cells had been dedifferentiated by culturing in the absence of doxycycline for 5 days. We started screening by adding each compound into individual wells of 96-well plates. Screening was done in the presence of 0.01 μ g/ml doxycycline, which induces small amounts of c-Myc, Klf4 and Sox9. We found that 280 compounds enhanced the intensity of the *Col11a2-EGFP* fluorescence. Among these 280 compounds, we excluded those that showed autofluorescence or contained a cycline structure, which is shared with doxycycline, from further analysis. Of the remaining compounds, 36 contributed to polygonal or round morphologies (Fig. 1B). Of these 36 compounds, real-time RT-PCR expression analysis showed that A-674563 (Fig. 1C) most

Fig. 2. Effects of A-674563 on primary chondrocyte differentiation and proliferation.

(A) Real-time RT-PCR expression analysis of the chondrocytic marker genes in primary chondrocytes treated with A-674563 at the indicated concentrations (μ M) for 3 days. Data shown as means $\pm$ s.d. *P < 0.05 and **P < 0.01 compared with DMSO by one-way ANOVA followed by the Dunnett post-hoc test. (n = 3 biological replicates). *Col11a2*, type XI collagen α 2 chain gene; *Col2a1*, type II collagen α 1 chain gene; and *Acan*, aggrecan gene.

(B) Primary chondrocytes were cultured in the absence or presence of various concentrations of A674563 for 3 days. Then, the cells were trypsinized, and the number of living cells were counted. Data are shown as means $\pm$ s.d. n.s. > 0.05 compared with DMSO by one-way ANOVA followed by the Dunnett post-hoc test (n = 3 wells).

(C) *Left*, immunoblot analysis of primary chondrocytes treated with or without A-674563 for 3 days. *Right*, signal intensities were analyzed with Image Quant TL (GE). Data are shown as means $\pm$ s.d. *P < 0.05 by Unpaired t-test (n = 3 wells).



substantially increased the expression level of *Col11a2* mRNA in 290-2-14 iChon cells.

3.2. A-674563 inhibited degradation of Sox9 and increased transcription of chondrocytic marker genes in mouse primary chondrocytes

The addition of A-674563 increased the mRNA expression of chondrocytic markers such as *Col11a2*, *Col2a1* and *Acan* in mouse primary chondrocytes in a dose-dependent manner (Fig. 2A). At the same time, A-674563 concentration did not significantly affect the number of cells (Fig. 2B). These results suggest that A-674563 enhances the expression of chondrocyte markers without compromising cell numbers, at least up to 0.3 μ M.

Interestingly, the addition of A-674563 did not increase the expression level of Sox9 mRNA (Fig. 2A), but did increase the amount of Sox9 protein (Fig. 2C). Since the transcriptions of *Col11a2*, *Col2a1* and *Acan* are activated by Sox9 [12–15], we speculated that the increased amount of Sox9 protein was responsible for the A-674563-mediated maintenance of chondrocytic characters. The discrepancy in mRNA and protein expression levels of Sox9 suggested that A-674563 inhibits the degradation of Sox9 protein. This suggestion was confirmed by additional experiments that showed A-674563 decreased reduction of Sox9 protein levels in primary chondrocytes after treatment with cycloheximide, an inhibitor of protein synthesis (Fig. 3A). We then treated mouse primary chondrocytes with bafilomycin (lysosomal inhibitor) or MG132 (proteasomal inhibitor) in the presence or absence of A-674563. The amount of Sox9 protein was increased by treatment with MG132 but not with bafilomycin in the absence of A-674563, suggesting that Sox9 is degraded through the proteasomal pathway (Fig. 3B). The increased amount of Sox9 protein caused by A-674563 was not changed by treatment with bafilomycin but further increased by treatment with MG132 (Fig. 3B). These results suggest that A-674563 and MG132 have additive effects towards the inhibition of Sox9 degradation.

3.3. Transcriptome analysis revealed that A-674563 increased *Usp29* expression

A-674563 has been reported to inhibit the Akt1 signaling pathway [16]. We analyzed the effects of Akt on chondrocytes by knocking down *Akt1*. We transfected mouse primary chondrocytes with two types of siRNAs corresponding to different stretches of *Akt1* mRNA. The transfection of *Akt1-a* or *Akt1-b* siRNA reduced the expression levels of Akt and phosphorylated Akt, but did not increase the amount of Sox9 (Fig. 4A). These results suggest that A-674563 promotes the chondrocytic phenotype through other mechanisms than down-regulation of the Akt pathway.

To get some insight into the mechanism, we performed transcriptome analysis. RNAs were extracted from mouse primary chondrocytes treated with A-674563 or vehicle and subjected to RNA sequencing analysis (Fig. 4B). The expression levels of

chondrocyte marker genes such as *Col2a1*, *Col9a1*, *Col9a2*, *Col9a3*, *Col11a1*, *Col11a2* and *Acan* were elevated in primary chondrocytes treated with A-674563 (Fig. 4C), confirming that the experiments were performed appropriately. On the other hand, the expression level of *Col1a1* was not changed. Furthermore, we found that the expression of the gene encoding ubiquitin-specific peptidase 29 (*Usp29*) was dramatically increased by treatment with A674563 (Fig. 4B). Real-time RT-PCR analysis confirmed that the addition of A674563 to primary chondrocyte culture increased the expression of *Usp29* in a dose-dependent manner (Fig. 4C).

4. Discussion

Although ACI is performed for the treatment of articular cartilage damage, their effectiveness is limited due to the dedifferentiation of chondrocytes in culture. Several factors have been reported to inhibit the dedifferentiation and improve treatment. For example, histone deacetylase (HDAC) increases type II collagen expression by suppressing the transcription of Wnt-5a during chondrocyte dedifferentiation [17]. Likewise, the inhibition of cathepsin K expression contributes to maintaining the chondrogenic phenotype in expansion culture [18]. In addition, the inhibition of the Rho family GTPase ROCK also prevents chondrocytes from losing their phenotype [19]. The present study adds another candidate compound that preserves chondrocytic characters, A-674563, and thus contributes to future the development of culture conditions that achieves chondrocyte expansion without chondrocyte dedifferentiation.

We established in this study a high-throughput screening system for compounds that enhance the expression of chondrocyte markers using a previously described chondrocyte-specific reporter cell line, 290-2-14 iChon cells [8]. This screening system identified A-674563 as capable of activating a reporter gene. Importantly, A-674563 increased the expression of chondrocyte markers in primary chondrocytes, demonstrating that this high-throughput system is effective at finding molecules that can enhance chondrocytic character.

The results suggested that A-674563 enhances the expression of chondrocytic marker genes by inhibiting the degradation of Sox9 protein. We found that Sox9 protein is degraded mainly through the proteasomal pathway, which is consistent with previous reports [20,21].

A-674563 has been shown to down-regulate Akt pathways [16], whereas our Akt knockdown experiment did not increase amount of Sox9 protein. Activation of Akt has been known to suppresses catabolic processes such as autophagy [22,23]. On the other hand, our results suggested that A-674563 inhibit degradation of Sox9 and that Sox9 is degraded mainly through proteasomal pathway. These findings and results collectively suggest that A-674563 inhibits degradation of Sox9 in chondrocytes through mechanisms other than downregulation of Akt signals, at least in the culture condition we employed.

To gain some insights into the mechanism through which A-

Fig. 4. Possible mechanisms of A-674563 action on chondrocytes.

(A) Primary chondrocytes were transfected with two types of siRNA (*Akt1-a* or *Akt1-b* siRNA), which correspond to different stretches of *Akt1* mRNA. 3 days later, cell lysates were subjected to immunoblots for Akt, Sox9 and β -actin.

(B and C) The results of the RNA-sequencing transcriptome analysis of primary chondrocytes treated with vehicle or 0.3 μ M A-674563 for 3 days ($n = 3$). RPKM, reads per kilobase of exon per million sequence reads.

(B) Volcano plots showing changes in gene expression in primary chondrocytes treated with A-674563 versus those with vehicle. Each dot represents one gene. The log fold change in the RPKM in primary chondrocytes treated with A674563 versus that with vehicle is represented on the x-axis. The y-axis shows the log10 of the p value. A p value of 0.003 by the Welch *t*-test is indicated by dotted line. Arrow represents *Usp29* gene.

(C) Expression of chondrocyte maker genes, *Col1a1* and *Usp29*. Expression levels are indicated by RPKM. Error bars denote means $\pm$ s.d. *** $P < 0.01$ compared with DMSO by unpaired *t*-test.

(D) Real-time RT-PCR expression analysis of *Col9a1* and *Usp29* in primary chondrocytes treated with A-674563 at the indicated concentrations for 3 days. Data are shown as means $\pm$ s.d. *** $P < 0.01$ compared with DMSO by one-way ANOVA followed by the Dunnett post-hoc test ($n = 3$ biological replicates).

674563 inhibit degradation of Sox9, we performed transcriptome analysis and found that A-674563 increases the expression of *Usp29* in primary chondrocytes. The *Usp29* gene encodes ubiquitin-specific peptidase 29, which has been reported to deubiquitinate and stabilize Claspin [24] and p53 [25], increasing the amounts of these two proteins. Whether *Usp29* regulates the expression of Sox9 in a similar manner is for further investigation.

In summary, we established a high-throughput screening system and used it to identify A-674563 as a molecule that inhibits the degradation of Sox9 to increase the expression of chondrocytic markers. Transcriptome analysis revealed that A-674563 elevates *Usp29* expression. The mechanisms through which these molecules regulate the chondrocytic phenotype remain to be analyzed. This study can contribute to future development of culture conditions that expand chondrocytes without losing chondrocytic characters, which should improve the quality of ACI.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.bbrc.2017.11.180>.

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