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The effect of resveratrol on normal and osteoarthritic chondrocyte metabolism

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Background

Resveratrol is a polyphenolic compound commonly found in the skins of red grapes. Sirtuin 1 (SIRT1) is a human gene that is activated by resveratrol and has been shown to promote longevity and boost mitochondrial metabolism. We examined the effect of resveratrol on normal and osteoarthritic (OA) human chondrocytes.

Methods

Normal and OA chondrocytes were incubated with various concentrations of resveratrol (1 μM, 10 μM, 25 μM and 50 μM) and cultured for 24, 48 or 72 hours or for six weeks. Cell proliferation, gene expression, and senescence were evaluated.

Results

SIRT1 was significantly upregulated in normal chondrocytes with resveratrol concentrations of 25 μM and 50 μM on both two- (2D) (both p = 0.001) and three-dimensional (3D) cultures (p = 0.008 and 0.001, respectively). It was significantly upregulated in OA chondrocytes treated with 10 μM, 25 μM and 50 μM resveratrol on 2D cultures (p = 0.036, 0.002 and 0.001, respectively) and at 50 μM concentration on 3D cultures (p = 0.001). At 72 hours, the expression of collagen (COL)-10, aggrecan (AGG), and runt-related transcription factor 2 (RUNX2) was significantly greater in both 25 μM (p = 0.011, 0.006 and 0.015, respectively) and 50 μM (p = 0.019, 0.004 and 0.002, respectively) resveratrol-treated normal chondrocyte cultures. In OA chondrocytes, expression of COL10 and RUNX2 was significantly greater in 25 μM (p = 0.004 and 0.024) and 50 μM (p = 0.004 and 0.019) cultures at 72 hours on 3D cultures.

Conclusions

At concentrations of 25 μM and/or 50 μM, resveratrol treatment significantly upregulates SIRT1 gene expression in normal and osteoarthritic chondrocytes. Resveratrol induces chondrocytes into a hypertrophic state through upregulation of COL1, COL10, and RUNX2.

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Article focus

- The effect of resveratrol on normal and osteoarthritic (OA) human chondrocytes

Key messages

- At concentrations of 25 μM and/or 50 μM, resveratrol treatment significantly upregulates SIRT1 gene expression in normal and osteoarthritic (OA) chondrocytes

Strengths and limitations

- Resveratrol treatment significantly upregulates SIRT1 gene expression in normal and osteoarthritic (OA) chondrocytes
- Resveratrol induces chondrocytes into a hypertrophic state through upregulation of COL1, COL10, and RUNX2

Introduction

Resveratrol (trans-3,4',5-trihydroxystilbene) is a polyphenolic compound commonly found in the skins of red grapes and red wine. Resveratrol has anti-inflammatory, anti-oxidant, and anti-ageing properties, and numerous studies have documented its neuro-, cardio-, and chondroprotective effects.[1-3] It has been suggested that the cardioprotective benefits of resveratrol are the cornerstone of the 'French Paradox', a diet

consisting of high saturated fat and regular red wine consumption that has been associated with a low risk of coronary artery disease.[4] Consumption or administration of resveratrol appears to mimic the effects of calorie restriction, which has long been identified as a mechanism of lifespan extension.[5] Increased longevity due to calorie restriction has been documented in organisms such as yeast, worms, flies, and mammals, and credited largely to decreased activity of insulin-like growth factor (Igf) insulin and target of rapamycin (TOR) signalling pathways.[6] Modulation of these pathways causes a variety of downstream effects, including altered metabolism[7] and the decline of age-related diseases.[8]

The precise molecular mechanism, by which resveratrol acts, is still a subject of debate. However, one effect of resveratrol, activation of the sirtuin1 (SIRT1) gene, appears to improve cell survival and metabolism[9-11] and increase longevity of mammalian cells under calorie restriction.[11-15] Cartilage is avascular tissue with a limited potential for repair and regeneration in response to injury or disease. In particular, chondrocytes have both a low proliferative capacity and cellular metabolism.[16] Because chondrocytes are solely responsible for maintaining cartilage tissue homeostasis, cell death and subsequent attrition of the extracellular matrix (ECM) can dramatically alter the structure and function of joints and result in the development of diseases such as osteoarthritis (OA).[17] Processes such as cellular ageing, senescence and chondrocyte apoptosis have been largely implicated in the initiation and progression of cartilage degradation,[18,19] which is characterised by mitochondrial dysfunction and depletion of cellular energy stores.

The purpose of our investigation was to examine the effect of resveratrol on osteoarthritic and normal human chondrocyte proliferation, senescence, and ECM gene activation. We hypothesised that resveratrol may prevent chondrocyte senescence and ageing, and increase cell proliferation and cartilage-specific gene expression.

Materials and Methods

All patients gave informed consent prior to inclusion. The study was authorised by the local ethical committee and was performed in accordance with the Ethical standards of the 1964 Declaration of Helsinki as revised in 2000.

Chondrocyte isolation and culture. Normal human chondrocytes were obtained from a commercially available non-transduced normal human chondrocyte cell system (Clonetics-Poietics, Walkersville, Maryland). Chondrocytes were passaged up to Passage 15 and were maintained at 37°C in humidified air containing 5% carbon dioxide during all experiments. Chondrocytes were grown in Dulbecco's Modified Eagle Medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic (Life technologies, Carlsbad, California). Adult human osteoarthritic articular cartilage samples (n = 2) were obtained from donors with OA undergoing total knee replacement. The OA chondrocyte isolation procedure was carried out under institutional review board approval. Using 'normal' cartilage on the periphery of the joint, OA chondrocytes were released from the cartilage matrix following overnight collagenase treatment (type II and type IV, at a final concentration of 1 mg/ml each) (Worthington, Freehold, New York) at 37°C in 5% carbon dioxide in 15 ml of DMEM/F-12 containing 10% FBS and 1% antibiotic antimycotic. Single cells were filtered through a nylon mesh filter and were collected by centrifugation, then washed three times with 10 ml phosphate buffered saline (PBS). The collected OA chondrocytes were then plated at a density of 1×10^5 cells/cm^2.

SIRT1 expression on two-dimensional culture. Normal and osteoarthritic chondrocytes were incubated in six-well plates (5×10^4 cells per well) for four days. Cells were then incubated with different concentrations of resveratrol (0, 1, 10, 25 and 50 µmol/l) (Sigma, St. Louis, Missouri) separately for 24 and 48 hours. Resveratrol liquid formulations were prepared using ethanol (Sigma) as a solvent. Concentrations were chosen to reflect the range of doses previously shown to induce differential cellular effects[20] and all conditions were run in triplicate. The expression of SIRT1 was analysed by real-time-reverse transcription polymerase chain reaction (real time RT-PCR) (Applied Biosystems, Foster City, California).

Cell proliferation assay. The effect of resveratrol on chondrocyte proliferation was determined using alamarBlue cell viability reagent assay (Invitrogen Life Technologies, Grand Valley, New York). AlamarBlue is a non-toxic aqueous fluorescent dye that does not affect cell phenotype, viability or cell proliferation.[21] Cells were incubated in 96-well plates (5×10^4 cells/well) for four days, to which resveratrol was added (to make a series of final concentrations: 1, 10, 25 and 50 µmol/l). Each concentration was applied to eight wells, whereas media supplemented with ethanol served as a control. After both a 24 hour and 48 hour culture, chondrocytes were supplemented with 10% (v/v) alamarBlue reagent and incubated for another three hours. Then, 100 µl of supernatant was read at 570/585 nm in a SpectraMax/M2 microplate reader (Molecular Devices, Sunnyvale, California). Cell numbers were determined from the standard curve.

Senescence-associated β-galactosidase activity. Senescence-associated β-galactosidase (SA-β-gal) activity was measured with a β-galactosidase staining kit (BioVision, Palo Alto, California). The protocol was conducted according to the manufacturer's instructions. Briefly, chondrocytes at day seven were treated with different concentrations of resveratrol (0, 1, 10, 25 or 50 µM) separately for 24 hours and 48 hours. Then chondrocytes were washed in PBS, fixed for ten to 15 minutes at room temperature with 0.5 ml of fixative solution, washed and incubated overnight at 37°C with the staining solution mix. Cells were observed under a microscope for development of

Table I. RT-PCR results for gene expression for normal and osteoarthritic (OA) chondrocytes following 72 hours of culture without resveratrol treatment. Type I collagen (COL1), type II collagen (COL2), type X collagen (COL10), Sox-9 (SOX9), and aggrecan (AGG) were chosen for their roles in chondrocyte metabolism and chondrocyte hypertrophy. Data is expressed as mean (SD).

	COL1	COL2	COL10	SOX9	AGG
Normal	1.2 (0.80)	11.8 (27.2)	1.1 (0.50)	1.3 (1.00)	1.4 (1.00)
OA	1.0 (0.30)	1.0 (0.13)	1.0 (0.22)	1.0 (0.16)	1.0 (0.15)

blue colour (total magnification × 200) and eight photomicrographs were obtained from each culture condition. A blue cell was defined as one having ≥ 50% area stained blue. In each image, two blinded observers quantified total cells, normal, and blue-stained cells. Cell counts from the two observers were averaged for analysis.[22] For both normal and OA chondrocytes, the percentage of senescent cells in 1 μM, 10 μM, 25 μM, and 50 μM resveratrol-treated groups were compared with the proportion of senescent cells in the control group.

Preparation and cell seeding of scaffolds (three-dimensional culture). Aqueous-derived silk fibroin scaffolds were prepared by adding 4 g of granular NaCl (particle size approximately 600 μm to 710 μm) into 2 ml of 6 wt% silk fibroin solution in disk-shaped Teflon (Savillex, Eden Prairie, Minnesota) containers. The containers were covered and left at room temperature for 24 hours, then immersed in water and the NaCl extracted for two days. The porosity of the aqueous-derived silk scaffolds was approximately 97% and the compressive strength and modulus were 60 KPa (SD 5) and 770 (SD 50) KPa, respectively.[23]

Chondrocytes (1×10^6 cells/scaffold) were seeded onto pre-wetted (DMEM/F12, overnight) scaffolds (5 mm diameter × 3 mm thick). The constructs were placed into 12-well plates. Cells were allowed to attach for 1.5 to 2 hours. The constructs were placed and incubated with different concentrations of resveratrol (0, 25 μmol/l and 50 μmol/l) in a humidified incubator at 37°C and 5% CO_2. All conditions were run in triplicate. Media were replaced every two to three days for six weeks.

Real-time RT-PCR. Total RNA from constructs (n = 3 per concentration) were extracted using Trizol reagent (Invitrogen, Carlsbad, California) and the isolated RNA concentration and quality was determined using a spectrophotometer. Quantitative real-time PCR assays for SIRT1, aggrecan (AGG), type X collagen (COL10), type I collagen (COL1), type II collagen (COL2), SOX9, runt-related transcription factor 2 (Runx2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts were carried out using gene-specific double-fluorescence-labeled probes in 7900 Sequence Detector (PE Applied Biosystems, Foster City, California). These probes and primers related to chondrogenesis and chondrocyte hypertrophy were obtained from Applied Biosystems as assays-on-demand gene expression products. Briefly, the RNA samples were reverse transcribed into cDNA using oligo (dT)-selection according to the manufacturer's protocol (High-Capacity cDNA Archive Kit; Applied Biosystems). Real-time PCR amplification was performed in a 384-well plate with a 13 ml reaction mixture. The thermal cycling conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles of 15 seconds of denaturation at 95°C and one minute of annealing and extension at 60°C. The comparative threshold (*Ct*) PCR cycle detection method (ΔΔ*Ct* method) that compares the differences in Ct values and treatment groups, was used to calculate the relative fold change in gene expression.

Statistical analysis. Data analysis was performed with SPSS Version 19 (SPSS Inc., Chicago, Illinois). The Shapiro–Wilk test was used to assess data normality. Normally distributed data were evaluated with a one-way analysis of variance (ANOVA) and Dunnett's two-sided *post-hoc* comparison. Data having a non-normal distribution were evaluated non-parametrically using Kruskal–Wallis and Bonferroni-corrected Mann–Whitney U tests. Significance was set at $p < 0.05$.

Results

Baseline gene expression. Expression of untreated chondrocytes after 72 hours in culture was used as the baseline gene expression; the mean (SD) is presented in Table I.

SIRT1 expression in 2D culture. In normal chondrocytes, there were no significant differences in SIRT1 expression when comparing control with resveratrol-treated groups at 24 hours ($p = 0.282$). However after 48 hours culture, a significant increase in SIRT1 expression was observed in groups treated with 25 μM and 50 μM resveratrol when compared with controls (both $p = 0.001$). There was no significant difference in SIRT1 expression between 1 μM and 10 μM resveratrol-treated groups and controls at 48 hours ($p = 0.997$ and $p = 0.892$, respectively) (Fig. 1).

In OA chondrocytes, a significant increase in SIRT1 expression was observed in cultures treated with 10 μM, 25 μM, and 50 μM resveratrol when compared with control groups after 24 hours ($p = 0.036$, 0.002 and 0.001, respectively). No significant difference was observed between 1 μM resveratrol-treated cultures and control cultures ($p = 1.00$). At 48 hours, SIRT1 expression was significantly elevated in 25 μM and 50 μM resveratrol-treated groups compared with control groups ($p = 0.011$ and $p = 0.014$, respectively). A significant difference was not observed in SIRT1 expression when comparing 1 μM and 10 μM resveratrol-treated cultures with control cultures at 48 hours ($p = 0.915$ and $p = 0.058$, respectively) (Fig. 1).

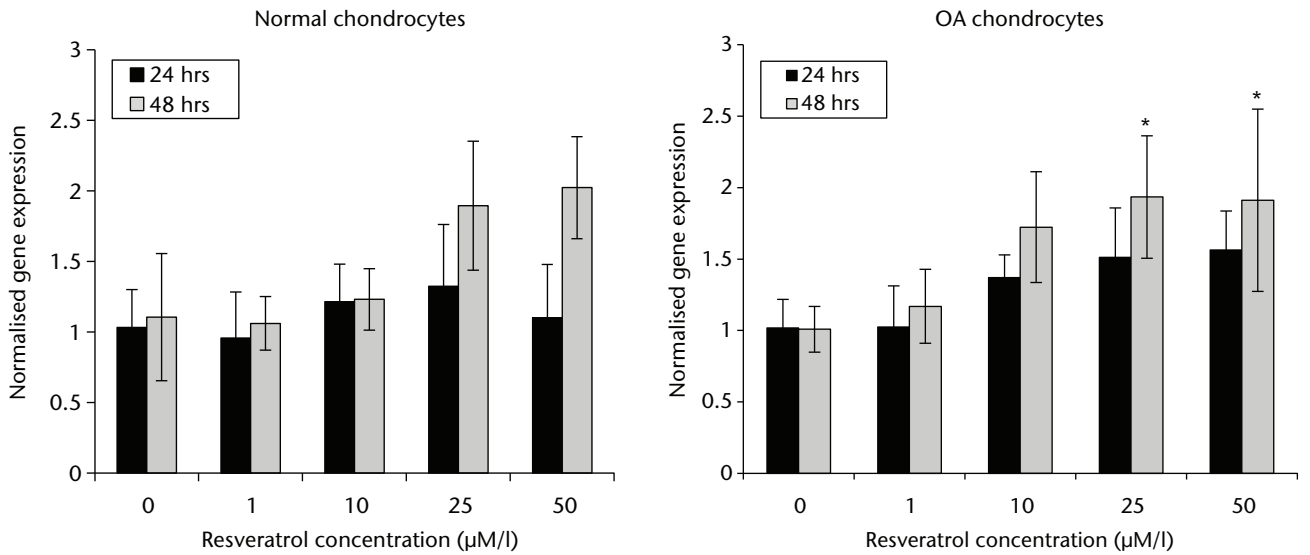

Fig. 1

Bar charts showing sirtuin 1 (SIRT1) expression in normal (left) and osteoarthritic (OA) chondrocytes (right) at 24 and 48 hours. Chondrocytes were incubated in six-well plates with different concentrations of resveratrol (0, 1 μM/l, 10 μM/l, 25 μM/l and 50 μM/l). Data are shown as the mean from eight samples with error bars denoting the standard deviation. * represents statistically significant differences compared with the control (0 μM) samples at the same incubation time. Transcript level was normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and untreated control within the linear range of amplication.

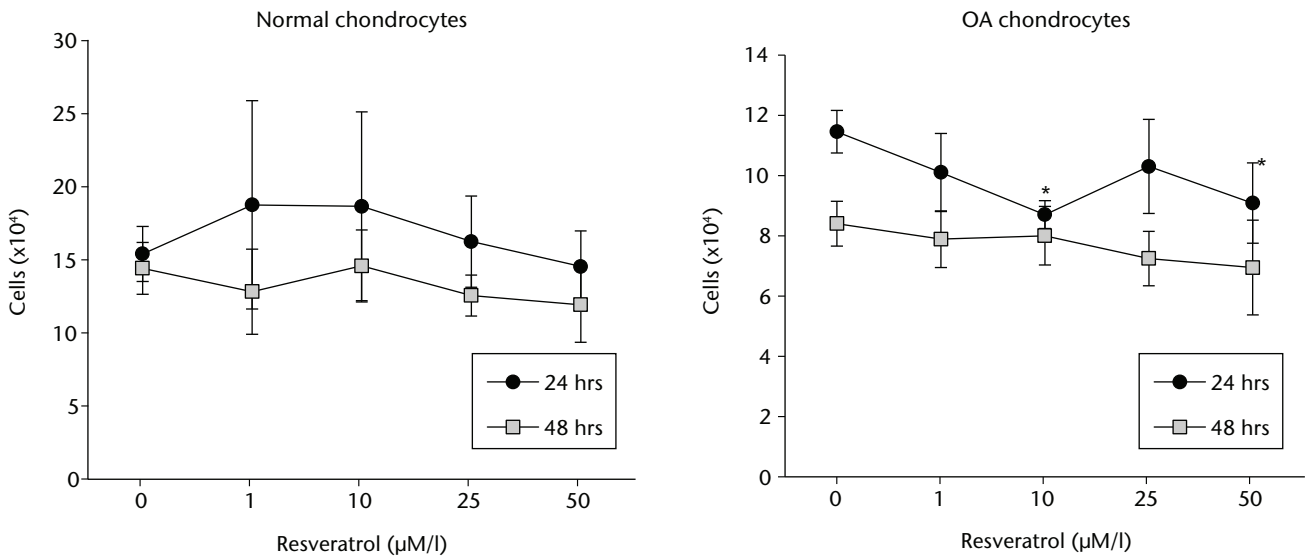

Fig. 2

Graphs of the results of alamar Blue cell proliferation assay, showing mean cell counts in each treatment group for normal (left) and osteoarthritic (OA) chondrocytes (right) at 24 and 48 hours. Cell counts were determined from the standard curve. Data are shown as the mean from eight samples, with error bars denoting the standard deviation. * represents statistically significant differences compared with the control (0 μM) samples at the same incubation time.

Cell proliferation. No significant difference in cell proliferation was observed between treatment conditions in normal chondrocytes at either 24 or 48 hours ($p = 0.497$ and $p = 0.104$, respectively) (Fig. 2). In OA chondrocytes, cell proliferation was significantly less in cultures treated with 10 μM or 50 μM resveratrol compared with controls at 24 hours ($p = 0.001$ and $p = 0.003$, respectively). However a significant difference between cultures was not observed at 48 hours ($p = 0.058$) (Fig. 2).

Cell senescence. In normal chondrocytes at 24 hours, the proportion of senescent cells was significantly greater in

Table II. The proportion of senescent cells in normal and osteoarthritic (OA) chondrocyte cultures at 24 and 48 hours. Chondrocytes were incubated in 12-well plates with different concentrations of resveratrol (0, 1 μmol/l, 10 μmol/l, 25 μmol/l and 50 μmol/l) in four samples for each experiment

	Resveratrol concentration (μM)								
Mean (SD) proportion of senescent cells (%)	**0**	**1**	**p-value***	**10**	**p-value***	**25**	**p-value***	**50**	**p-value***
24 HOURS									
Normal chondrocytes	41.0 (5.8)	33.0 (6.9)	0.148	36.1 (1.9)	0.509	56.4 (5.6)	0.003	40.5 (6.1)	1.00
OA chondrocytes	48.8 (8.2)	48.0 (4.9)	0.999	51.2 (5.3)	0.908	49.7 (3.0)	0.996	42.5 (2.8)	0.300
p-value	0.171	0.012		0.002		0.048		0.565	
48 HOURS									
Normal chondrocytes	30.6 (14.0)	34.9 (7.0)	0.844	37.9 (4.5)	0.503	44.4 (5.3)	0.076	42.9 (3.2)	0.123
OA chondrocytes	31.5 (2.2)	35.0 (4.3)	0.533	42.3 (3.7)	0.004	39.8 (3.6)	0.028	44.2 (5.0)	0.001
p-value	0.898	0.987		0.179		0.197		0.691	

* compared with control cultures (0 μM concentration). Analysed using independent *t*-test

cultures treated with 25 μM resveratrol compared with control cultures (56.4% (SD 5.63) *vs* 41.0% (SD 5.8), p = 0.003). Significant differences were not observed at any other concentrations. At 48 hours, the percentage of senescent cells did not differ significantly between control cultures and any of the treatment groups (Table II).

In OA chondrocytes at 24 hours, the percentage of senescent cells was in control cultures was not significantly different from the percentage of senescent cells in groups treated with 1 μM, 10 μM, 25 μM, or 50 μM resveratrol (Table I). However, compared with control cultures at 48 hours (31.52% (SD 2.15)), the percentage of senescent cells was significantly greater in cultures treated with 10 μM (42.29% (SD 3.66), p = 0.004), 25 μM (39.75% (SD 3.60), p = 0.028) and 50 μM (44.18% (SD 5.0), p = 0.001) (Table II).

Gene expression in 3D culture. In normal chondrocytes at 72 hours, expression of COL10 (p = 0.011), AGG (p = 0.006), and RUNX2 (p = 0.015) was significantly greater in cultures treated with 25 μM resveratrol when compared with control cultures. In 50 μM resveratrol-treated cultures, expression of SITR1 (p = 0.001), COL1 (p = 0.004), COL10 (p = 0.019), SOX9 (p = 0.010), AGG (p = 0.004) and RUNX2 (p = 0.002) was significantly greater than in control cultures; however, a difference was not observed with COL2 (p = 0.522).

At six weeks, expression of SIRT1 (p = 0.008), COL1 (p = 0.004), COL10 (p = 0.004), AGG (p = 0.010), and RUNX2 (p < 0.01) was significantly greater in cultures treated with 25 μM resveratrol when compared with control cultures but no difference was observed for COL2 (p = 0.150) or SOX9 (p = 0.150). In 50 μM resveratrol-treated cultures, expression of SIRT1 (p = 0.001), COL1 (p = 0.004), COL10 (p = 0.004), AGG (p = 0.037) and RUNX2 (p = 0.012) was significantly greater than in control cultures, but differences were not observed for COL2 or SOX9 (both p = 0.337) (Fig. 3).

In OA chondrocytes at 72 hours, cultures treated with 25 μM resveratrol exhibited expression of COL1 (p = 0.004), COL10 (p = 0.004), and RUNX2 (p = 0.024) that was significantly greater than control cultures, while expression of COL2 (p = 0.025) and SOX9 (p = 0.004) was significantly less when compared with control cultures. No difference was observed for SIRT1 (p = 0.820) and AGG expression (p = 0.262). In 50 μM resveratrol-treated cultures, expression of SIRT1 (P = 0.004), COL10 (p = 0.004) and RUNX2 (p = 0.019) was significantly greater compared with control cultures, while expression of COL2 (p = 0.004) and AGG (p = 0.037) was significantly less when compared with controls. Though not statistically significant, similar trends for COL1 and SOX9 were observed: expression of COL1 was greater compared with control cultures (p = 0.055) and SOX9 expression was less (p = 0.055).

At six weeks, in cultures treated with 25 μM resveratrol, expression of COL10 (p = 0.004) was significantly greater than control cultures while expression of COL2 (p = 0.004), SOX9 (p = 0.025), and RUNX2 (p = 0.004) was significantly less compared with controls. No differences were observed for SIRT1 (p = 0.728), COL1 (p = 0.873) and AGG (p = 0.262). In 50 μM resveratrol cultures, expression of SIRT1 (p = 0.001) and COL10 (p = 0.004) were significantly greater than control cultures while expression of COL1 (p = 0.016), COL2 (p = 0.004) and RUNX2 (p = 0.004) was significantly less compared with controls. Significant differences were not observed for SOX9 (p = 0.337) or AGG (p = 0.150) (Fig. 4).

Discussion

Resveratrol (trans-3,4',5-trihydroxystilbene) is a polyphenolic compound found in the skins of red grapes and red wine that has been shown to have anti-oxidant, anti-inflammatory, metabolic and anti-ageing properties.[24] However, it is increasingly understood that the specific effects of resveratrol are dependent on both dosage and cell type. *In vitro* resveratrol treatment of chondrocytes has been shown to suppress various IL-1β-induced inflammatory signaling cascades,[25-27] protect cells against apoptosis[26,28] and decrease advanced glycation end products and matrix metalloproteinase production.[29] *In vivo* intra-articular injections of resveratrol have demonstrated potential chondroprotective effects, particularly in animal models of OA.[30,31] The precise molecular mechanism by which resveratrol acts

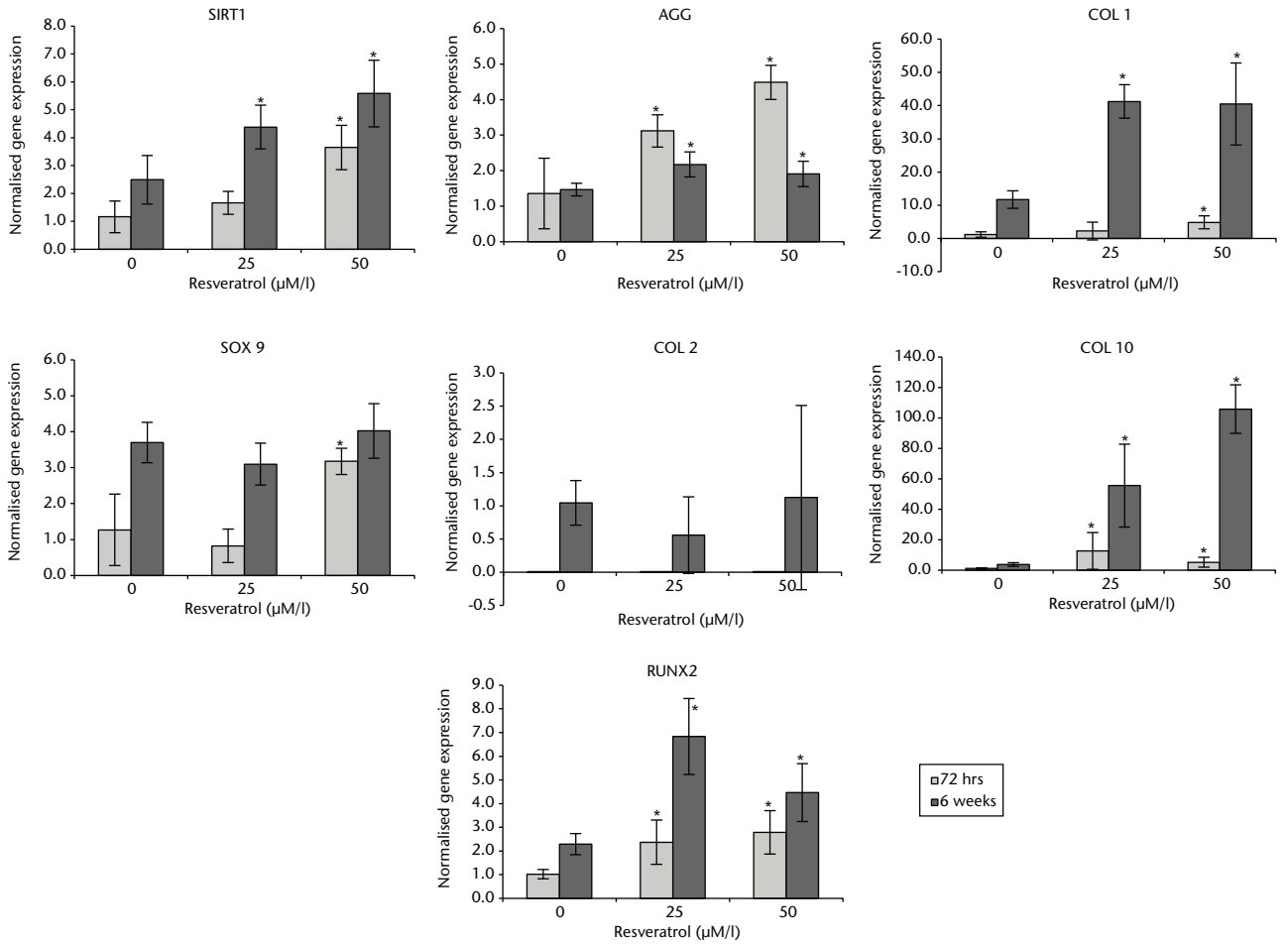

Fig. 3

Bar charts showing the expression of sirtuin 1 (SIRT1) and chondrogenesis-related genes by concentration of resveratrol in normal chondrocytes. Chondrocytes were cultured on scaffolds with different concentrations of resveratrol (0, 25 μM/l and 50 μM/l) up to six weeks. Data are shown as the mean from six samples with error bars denoting the standard deviation. * represents statistically significant differences compared with the control (0 μM) samples at the same incubation time. Transcript levels were normalized to GAPDH and untreated controls within the linear range of amplification. (AGG, aggrecan; COL, collagen; RUNX2, runt-related transcription factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase).

is not firmly established, but it is generally believed that the beneficial effects of resveratrol are due to the direct or indirect activation of SIRT1, a human gene shown to improve cell survival and increase longevity.[12,13]

SIRT1 activation has been correlated with the upregulation of the cartilage-specific genes COL2 and AGG,[9] while SIRT1 inhibition has been shown to result in gene expression characteristic of chondrocyte hypertrophy, including upregulation of COL10 in normal human chondrocytes.[10] This evidence led to our hypothesis that treatment with resveratrol might activate SIRT1 in normal and osteoarthritic chondrocytes, resulting in increased cellular proliferation, upregulation of cartilage-specific genes, and decreased cellular senescence.

The present study revealed three significant findings: 1) resveratrol concentrations of 25 μM and/or 50 μM significantly upregulated SIRT1 mRNA in both normal and osteoarthritic chondrocytes; 2) treatment with resveratrol induced gene expression characteristic of chondrocyte hypertrophy, suggesting that chondrocytes were progressing down an endochondral bone pathway in response to resveratrol treatment; and 3) OA chondrocytes treated with 10 μM, 25 μM, or 50 μM resveratrol for 48 hours showed significantly more senescence compared with control cultures. No significant difference in cell proliferation was observed in either normal or OA chondrocytes.

The upregulation of SIRT1 mRNA observed in both normal and OA chondrocytes following treatment with 25 μM and/or 50 μM resveratrol (Figs 1, 3 and 4) is consistent with previous studies in which resveratrol was identified as a small molecule activator of SIRT1.[32,33] Though the precise molecular link between SIRT1 and resveratrol is still being explored, the results of our investigation support the theory that resveratrol upregulates the expression of SIRT1 and may act in a dose-dependent manner.

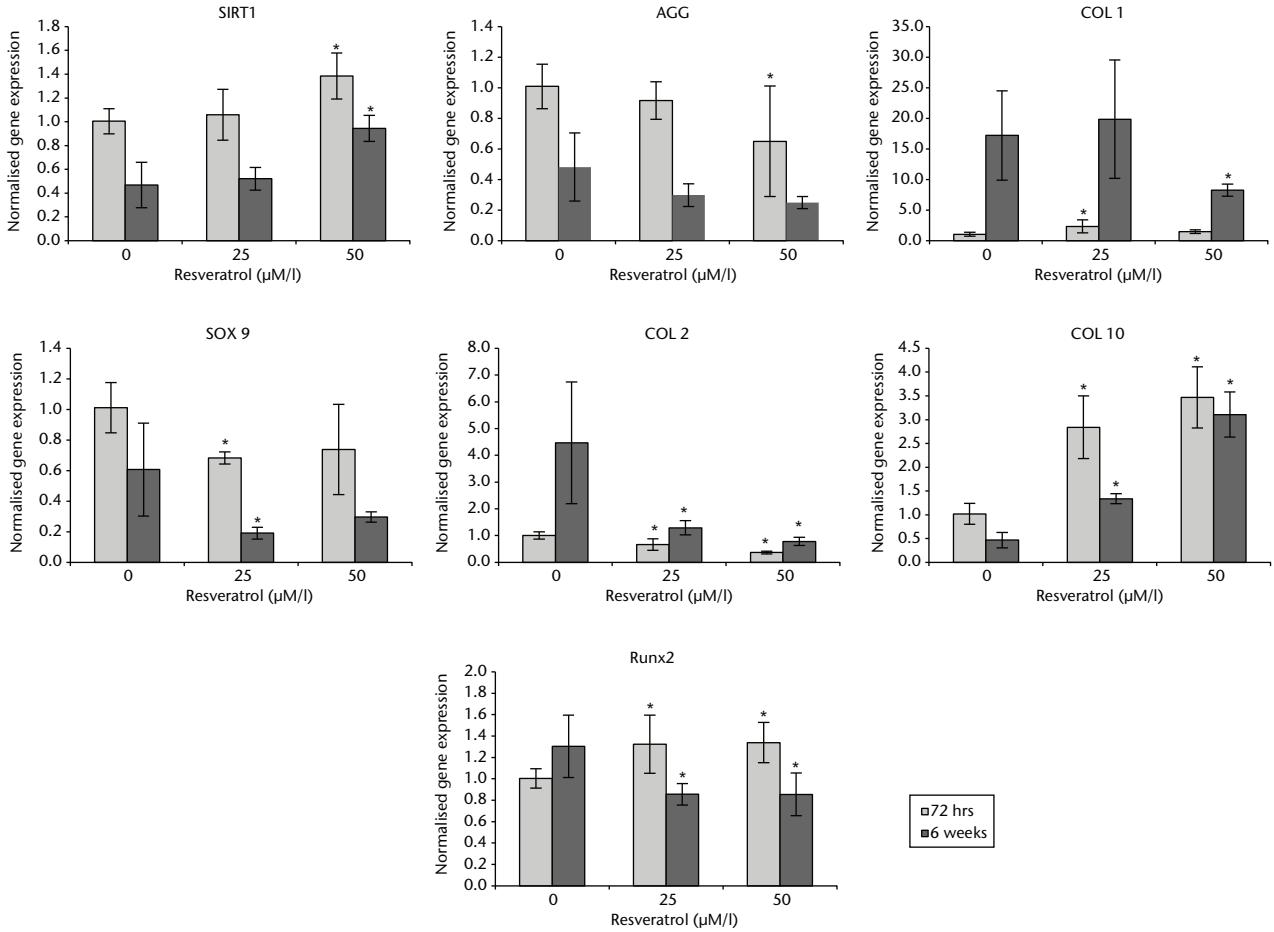

Fig. 4

Bar charts showing the expression of sirtuin 1 (SIRT1) and chondrogenesis-related genes by concentration of resveratrol in osteoarthritic chondrocytes. Chondrocytes were cultured on scaffolds with different concentrations of resveratrol (0, 25 and 50 μM/l) up to six weeks. Data are shown as the mean from six samples with error bars denoting the standard deviation. * represents statistically significant differences compared with the control (0 μM) samples at the same incubation time Transcript levels were normalized to GAPDH and untreated controls within the linear range of amplification. (AGG, aggrecan; COL, collagen; RUNX2, runt-related transcription factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase).

Recent investigations[9,11] have suggested that SIRT1 activation may protect cartilage from OA-like degeneration via inhibition of apoptosis and upregulation of cartilage-specific genes. Accordingly, consistent with the observed SIRT1 upregulation, we expected to see increased expression of SOX9, COL2, and AGG and decreased expression of COL1 and COL10. However, we found no evidence that resveratrol stimulated chondrogenic gene expression.

In normal chondrocytes treated with 25 μM and 50 μM resveratrol, there was significant upregulation of COL1, COL10, and RUNX2, all markers of hypertrophic progression in chondrocytes. This effect was seen at 72 hours and six weeks, with a more profound effect at the longer period (Fig. 3). The significant upregulation of hypertropic chondrocyte markers, including terminal differentiation marker RUNX2,[34] suggests that treatment with resveratrol induces normal chondrocytes to progress down the hypertropic chondrocyte lineage.

Similar trends were observed in OA chondrocytes. At 25 μM and 50 μM concentrations of resveratrol, expression of COL1, COL10, and RUNX2 was significantly greater, while expression of COL2, SOX9, and AGG was significantly lower at 72 hours. At six weeks, OA chondrocytes also showed significantly greater expression of COL10 and significantly less COL2, SOX9, and RUNX2 expression, which is consistent with reported SOX9 suppression indicative of chondrocyte hypertrophy.[35] As was the case with normal chondrocytes, the decrease in expression of SOX9, COL2, and AGG and the increase in expression of COL1 and COL10 in OA chondrocytes suggest a progression towards endochondral ossification and osteogenesis. However, unlike normal chondrocytes, RUNX2 expression in OA chondrocytes was significantly less in both 25 μM and 50 μM cultures when compared with controls (Fig. 4), possibly suggesting that OA chondrocytes were slower in progressing to the hypertrophic chondrocyte stage or had lost the capacity for osteogenic

differentiation. The aggregate of these results indicates that both OA and normal chondrocytes treated with 25 μM and 50 μM resveratrol exhibit changes in gene expression characteristic of osteochondral ossification, in which cellular hypertrophy eventually progresses to mineralisation and bone formation.[36]

Previous investigations have shown that resveratrol stimulates and promotes osteogenic and osteoblastic differentiation in human periodontal ligament cells,[37] murine-induced pluripotent stem cells[38] and mesenchymal stem cells (MSCs).[39,40] In particular, it has been demonstrated that activation of SIRT1 *via* resveratrol results in upregulation of RUNX2 in human MSCs,[37] and that resveratrol may antagonise osteoclast differentiation[41,42] and promote osteoblast formation.[43] These findings, coupled with our results, suggest there may be future roles for resveratrol in fracture healing.

Although we expected to see increased proliferation following resveratrol administration, no significant difference in cell proliferation was observed for normal chondrocytes treated with resveratrol, and OA chondrocytes treated with resveratrol demonstrated significantly less cell proliferation after 24 hours (Fig. 2). Previous studies have demonstrated that resveratrol inhibits cell proliferation in synoviocytes,[44] retinal pigment epithelial cells[45] and endothelial cells,[46] while other investigations reported increased cell proliferation in endothelial progenitor cells,[47] human bone marrow-derived MSCs[48] and osteoblastic MC3T3-E1 cells.[49] In the present study, resveratrol did not enhance or increase cell proliferation in normal or OA chondrocytes, suggesting that the proliferative effects of resveratrol may be dependent on cell type. Similarly, despite the increase in SIRT1 upregulation, we observed that cell senescence in OA chondrocytes was significantly increased in cultures treated with 10 μM, 25 μM, or 50 μM resveratrol (Table I). Increased senescence following resveratrol treatment has been recently observed in multiple strains of primary human fibroblasts and is thought to be mediated by p38 activity.[50] However, given that SIRT1 activation has been shown to prevent growth arrest and senescence[51] it is was particularly interesting that OA chondrocytes simultaneously demonstrated both SIRT1 upregulation and cellular senescence. Our results showed that normal chondrocytes had greater upregulation of SIRT1 compared with OA chondrocytes, a finding that had been previously documented.[52] It is plausible then, that abnormal cellular metabolism characteristic of OA chondrocytes resulted in irreversible damage that could not be repaired, even after SIRT1 upregulation.

As with any laboratory investigation, this study was subject to inherent limitations. A 3D cell scaffold was used in attempt to mimic the environment present in *en bloc* cartilage; however, these conditions are not equivalent to the *in vivo* joint environment. Our observations of significant SIRT1 upregulation and expression of markers of chondrocyte hypertrophy following treatment with resveratrol, suggest the potential for resveratrol to be used in bone healing models.

At 25 μM and/or 50 μM concentrations, resveratrol treatment significantly upregulates SIRT1 gene expression in both normal and osteoarthritic chondrocytes. It appears that resveratrol also induces normal and osteoarthritic chondrocytes into a hypertrophic state through upregulation of COL1, COL10, and RUNX2. In osteoarthritic chondrocytes, 10 μM, 25 μM, and 50 μM concentrations of resveratrol increased cellular senescence. The addition of resveratrol to chondrocyte cultures had minimal effect on cell proliferation.

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- H. J. Kim: Data collection, Data analysis, Writing the paper
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- None declared