Biochemical and Biophysical Research Communications 406 (2011) 59–63

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

14-3-3η inhibits chondrogenic differentiation of ATDC5 cell

Hee Eun Yoon, Ki Soon Kim, Ick Young Kim *

Laboratory of Cellular and Molecular Biochemistry, School of Life Sciences and Biotechnology, Korea University, 1, 5-Ka, Anam-Dong, Sungbuk-Ku, Seoul 136-701, Republic of Korea

ARTICLE INFO

Article history:
Received 4 January 2011
Available online 1 February 2011

Keywords:
Chondrogenesis
14-3-3η
Joint inflammation
ATDC5 cells

ABSTRACT

It was previously shown that 14-3-3η is overexpressed in the synovial fluid of patients with joint inflammation, which is often associated with growth failure. In this study, we investigated the role of 14-3-3η in chondrogenesis using ATDC5 cells. Upon treatment with TNF-α, cells overexpressed 14-3-3η with inhibition of chondrogenesis. Chondrogenesis was also inhibited by overexpression of 14-3-3η without TNF-α treatment, whereas silencing of 14-3-3η promoted chondrogenic differentiation. Further, G1 phase arrest was inhibited by overexpression of 14-3-3η. In summary, we suggest that 14-3-3η plays a regulatory role in chondrogenic differentiation.

1. Introduction

The 14-3-3 proteins are a family of regulatory molecules and are found in all eukaryotes. So far, seven isoforms (β, ε, ζ, η, θ, γ and σ) have been confirmed in mammals [1,2]. Distinct isoforms of 14-3-3 proteins have been reported to play essential roles in regulating cell differentiation, proliferation and transformation, and also interact with over 200 target proteins related to signal transduction, cell cycle control, cell growth, survival, and apoptosis [3,4]. Interestingly, despite high sequence homologies and structural similarities between 14-3-3 isoforms, the pathways in which these proteins participate are considerably divergent [5]. A recent study reported that 14-3-3η and γ are found in the serum and synovial fluid of patients with joint inflammation. Especially, 14-3-3η was significantly overexpressed in juvenile rheumatoid arthritis (JRA) [6]. JRA is a chronic inflammatory disease often associated with growth impairment. Linear growth occurs by endochondral ossification of long bones. Chondrogenesis comprises proliferation, hypertrophy of growth plate chondrocytes, and synthesis of the cartilaginous matrix [7,8]. These conditions are required for expression of marker proteins related to chondrogenesis. The transcription factor Sox9 plays an essential role in the chondrogenic differentiation pathway by regulating the transcription of cartilage-specific extracellular matrix (ECM) molecules, including collagen type II and aggrecan. In early chondrogenesis, ECM proteins are highly expressed [9,10]. Some studies have reported that proinflammatory cytokines such as IL-1 and TNF-α decrease chondrocyte proliferation of the growth plate [11] and also markedly reduce Sox9 expression [12,13].

Cell cycle factors appear to play important role in the control of chondrocyte proliferation and differentiation. G1 phase arrest of the cell cycle is a prerequisite for chondrogenic differentiation [14,15]. 14-3-3 proteins regulate the cell cycle via interaction with several checkpoints [16].

So far, the role of 14-3-3η in chondrogenesis is unknown. A recent study reported that 14-3-3η selectively regulates differentiation of neurons and astrocytes. However, it is not demonstrated that 14-3-3η is affected for differentiation mechanism in neurons and astrocytes [17].

In this study we focused on the possible role of 14-3-3η in the regulation of chondrogenic differentiation using the ATDC5 cell line, which is a useful *in vitro* model for chondrogenic differentiation and allows study of ossification at the growth plate [18].

2. Materials and methods

2.1. Materials

Mouse recombinant TNF-α was obtained from R&D systems (Minnesota, USA). TNF-α antagonist was purchased from ANASPEC (San Francisco, USA). Anti-HA antibody, anti-14-3-3η antibody, anti-Sox9 antibody, anti-cyclin D1 antibody and anti-cyclin B1 antibody were purchased from Santa Cruz Bioechnology (California, USA). Anti-cyclin D3 antibody and anti-p21 antibody were obtained from BD bioscience (San Diego, USA). Anti-Collage type II antibody was purchased from Millipore (Massachusetts, USA).

2.2. Cell culture

The mouse embryonic carcinoma-derived chondrogenic cell line ATDC5 was purchased from Cell Bank, RIKEN BioResource Center (Tsukuba, Japan). ATDC5 cells were cultured in medium

* Corresponding author. Fax: +82 2 3290 3449.
E-mail address: ickkim@korea.ac.kr (I.Y. Kim).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.01.107

consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Invitrogen) containing 5% heat-inactivated fetal bovine serum, penicillin (100 U/ml) 10 μg/ml of human transferrin (Roche Diagnostics), and 3×10^{-8} M sodium selenite (Sigma–Aldrich) at 37 °C in a 5% CO_2 humidified atmosphere. For induction of chondrogenesis, human recombinant insulin (Sigma, 10 μg/ml) was added to the medium [19].

2.3. Quantitative real-time PCR

Total RNA was isolated from ATDC5 cells using TRIzol reagent(-Sigma–Aldrich), and 1 μg of total RNA was reverse-transcribed in a final volume of 20 μl using AccuPower CycleScript RT PreMix (dT20)(Bioneer, Korea). Quanitative real-time PCR was carried out using 150 ng/well of cDNA template, the gene specific primers *Col2a1*, 5′-GAAAAACTGGTGGAGCAGCAAGAGC-3′ and 5′-CAATAAT GGGAAGGCGGGAGGTC-3′; *sox9*, 5′-AGTTACGGCATCAGCAGCAC-3′ and 5′-CAGCTGCTCCGTCTTGATGT-3′; *18s*, 5′-GTAACCCGTTGAAC C-3′ and 5′-CCATCCAATCGGTAGTAGCG-3′ and SYBR Premix Ex Taq (Takara) in a 384-well plate (Roche). The products were analyzed using a LightCycle 480 real-time PCR system (Roche). Relative gene expression was normalized to the 18s reference gene. Analysis of the data was repeated three times.

2.4. Western blot analysis

Cell lysate prepared as described previously [20] was subjected to SDS–PAGE and transferred to PVDF membrane. Immunoreactive proteins were detected by SuperSignal West Pico enhanced chemical luminescence (ECL) (Thermo Scientific).

2.5. Flow cytometry

ATDC5 cells transfected with pcDNA3.1 or HA-14-3-3η [21], were dispersed into single-cell suspensions by treatment with trypsin/EDTA. The dispersed cells were washed three times with PBS and fixed with 70% ethanol at −30 °C for 2 h. Then, the cells were incubated with 1 mg/ml of RNase A in PBS at 37 °C for 30 min and stained at room temperature for 5 min with 50 μg/ml of propidium iodide (PI) prepared in PBS. DNA content was determined by flow cytometry using a FACScalibur flow cytometer (BD bioscience), and the data were analyzed with CellQuest software (Becton Dickinson).

2.6. Alcian blue staining

Cells were cultured in 12-well plates at a density of 1.5×10^5 cells/well, followed by transfection with pcDNA3.1 or HA-14-3-3η. Chondrogenic differentiation of ATDC5 was induced by insulin treatment for 2 days. The cells were rinsed with PBS three times and then fixed with 95% methanol for 5 min at −20 °C. Cells were stained with 0.1% Alcian blue 8GX (Sigma) in 0.1 M HCl as previously described [22]. Absorbance of the extracted dye was measured using a spectrophotometer at 655 nm. Cartilage nodule formation was observed by phase contrast microcopy (Olympus CKX41) at day 2.

2.7. siRNA experiments

siRNAs targeting mouse 14-3-3η (NM_011738) (5′-CAAA-CAAGCCTTCGATGATGC TATA-3′) were purchased from Invitrogen. Stealth RNAi negative control (Invitrogen) was used as a control. Cells were transfected with siRNA using lipofectamine RNAiMAX reagent (Invitrogen) for 5 h in OPTI-MEM medium (Gibco BRL). Then, the medium was replaced with growth medium.

3. Results and discussion

3.1. Inhibition of chondrogenesis by TNF-α increases 14-3-3η expression

JRA is associated with growth impairment. In mammals, growth impairment results from disordered longitudinal bone growth, which is associated with abnormal growth plate chondrogenesis [23]. A recent study reported that 14-3-3η isoform is easily detectable in the synovial fluid of patients with inflamed joints [6]. IL-1 and TNF-α are also significantly elevated in chronic inflammatory illnesses such as JRA [24].

We first examined whether or not 14-3-3η expression is increased by TNF-α, a proinflammatory cytokine, during chondrogenesis of ATDC5 cells. For this, chondrogenesis of the cells was induced by insulin, after which differentiation was determined based on the expression level of either Sox9 or Collagen type II (Fig. 1). 14-3-3η expression was increased during chondrogenesis, and it was further increased by TNF-α treatment even without insulin treatment (Fig. 1A). However, chondrogenic differentiation was inhibited by TNF-α treatment. The increased expression of 14-3-3η by TNF-α was nullified by treatment with WP9QY, a TNF-α antagonist [25]. Furthermore, chondrogenesis inhibited by TNF-α was restored by the antagonist treatment (Fig. 1B). These results indicate that TNF-α affects the expression of 14-3-3η during chondrogenic differentiation of ATDC5 cells.

3.2. Overexpression of 14-3-3η inhibits chondrogenesis of ATDC5 cells

We next evaluated the effect of 14-3-3η on the differentiation of ATDC5 cells. For this, ATDC5 cells were transfected with 14-3-3η, after which we examined the expression level of Sox9

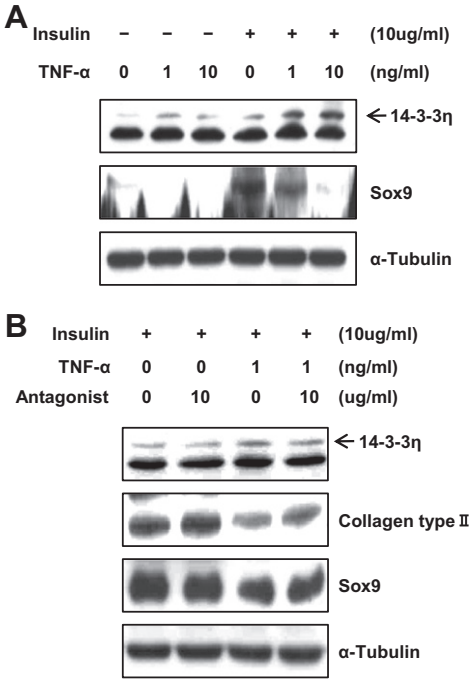

Fig. 1. Expression of 14-3-3η is increased by TNF-α during chondrogenesis. (A) ATDC5 cells were treated with different concentrations of TNF-α and cultured with or without insulin for 2 days. The expression levels of 14-3-3η and Sox9 were measured by Western blot analysis. α-Tubulin was used as a protein loading control. (B) ATDC5 cells were treated with TNF-α after pretreatment with or without the TNF-α antagonist WP9QY. Cells were then induced with insulin for 2 days, after which cell lysates were analyzed by immunoblotting with anti-14-3-3η, anti-collagen type II, anti-Sox9, or anti-α-tubulin antibodies.

and Collagen type II on day 2. Quantitative real-time PCR analysis showed that the overexpression of 14-3-3η reduced the mRNA levels of collagen type II (*col2a1*) and Sox9 (*sox9*) (Fig. 2A). Protein levels were also decreased in 14-3-3η-overexpressed ATDC5 cells (Fig. 2B). Further, formation of cartilage nodules was reduced in 14-3-3η-transfected cells compared to mock control cells (Fig. 2C). These data indicate that 14-3-3η inhibits the chondrogenesis of ATDC5 cells.

3.3. Silencing of 14-3-3η promotes chondrogenic differentiation of ATDC5 cells

Next, we determined whether or not the down-regulation of 14-3-3η via transient introduction of specific siRNA affects the chondrogenic differentiation of ATDC5 cells. As shown in Fig. 3A and B, both the mRNA and protein levels of collagen type II (*col2a1*) and Sox9 (*sox9*) were high in 14-3-3η-silenced cells during chon-

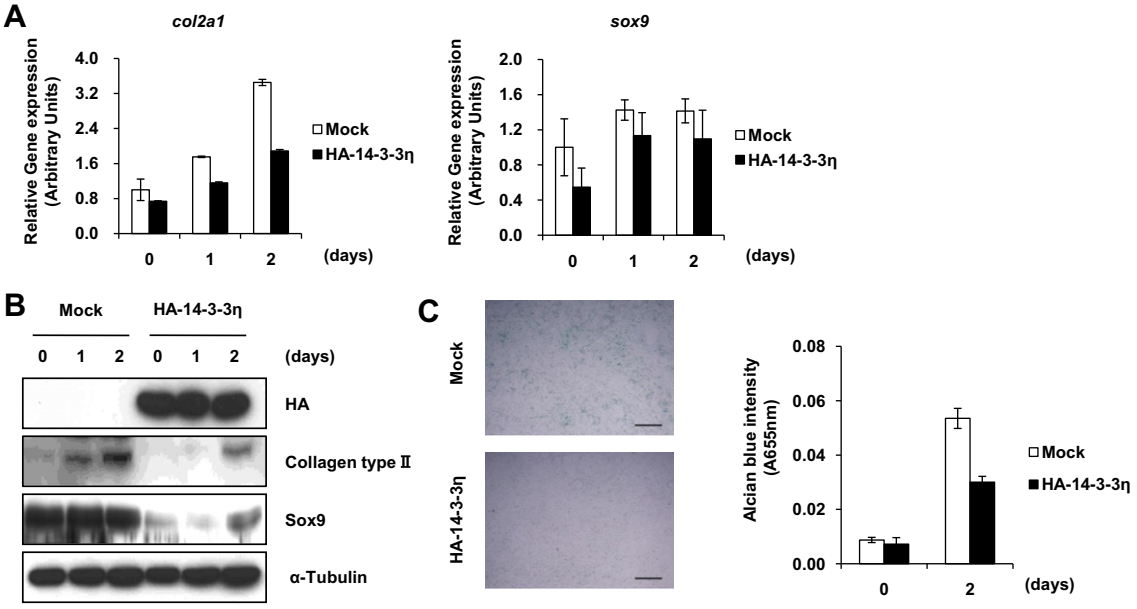

Fig. 2. Chondrogenesis is inhibited by 14-3-3η overexpression in ATDC5 cells. ATDC5 cells were seeded in 60 mm plates at a density of 4×10^5 cells/well. These cells were transiently transfected with pcDNA3.1 control vector (mock) or expression vector containing HA-14-3-3η. Cells were treated with or without insulin for 2 days. (A) Total RNA isolated from cells was subjected to real-time qPCR analysis for *collagen type II* and *sox9* mRNA. (B) Cell lysates from ATDC5 cells transfected with pcDNA3.1 control vector (mock) or HA-14-3-3η were analyzed by immunoblotting with anti-HA, anti-collagen type II, anti-Sox9, or anti-α-tubulin antibodies. (C) ATDC5 cells were transfected with pcDNA3.1 control vector (mock) or HA-14-3-3η and induced by insulin for 2 days. Then, the cells were stained with Alcian blue. Extraction of stained cells was quantified by measuring the absorbance at 655 nm. (–, 200 μm).

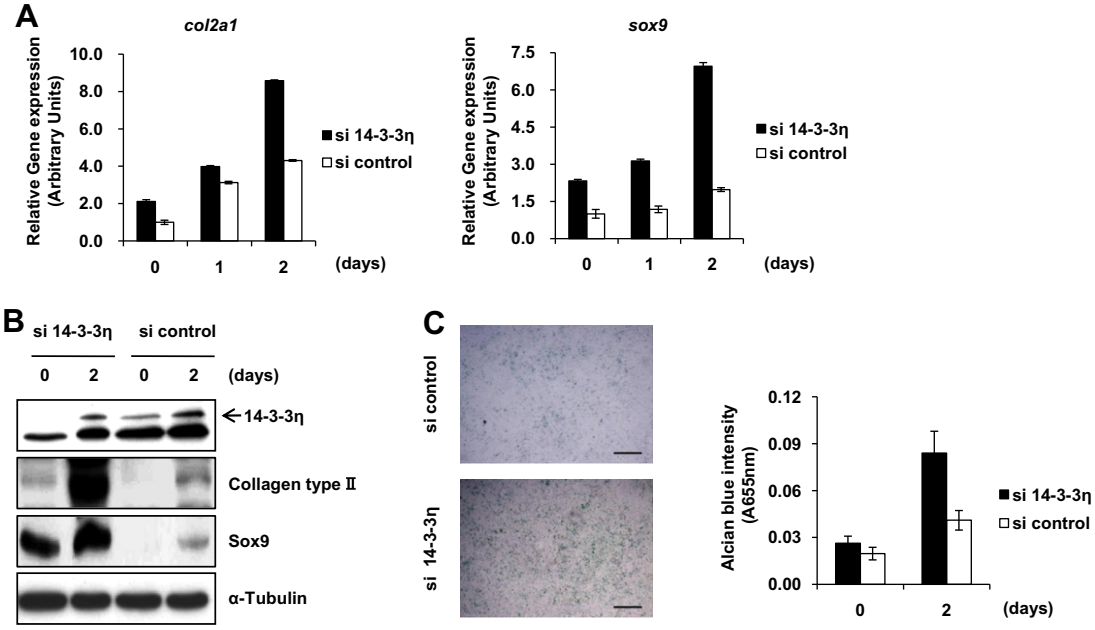

Fig. 3. Silencing of 14-3-3η promotes chondrogenic differentiation in ATDC5 cells. ATDC5 cells were cultured in 60 mm plates at a density of 2×10^5 cells/well. These cells were then transfected with control or specific siRNA for 14-3-3η for 48 h, and chondrogenic differentiation was induced by insulin for 2 days. (A) Total RNA isolated from cells was subjected to real-time qPCR analysis for *collagen type II* and *sox9* mRNA. (B) Cell lysates were analyzed by immunoblotting with anti-14-3-3η, anti-collagen type II, anti-Sox9, or anti-α-tubulin antibodies. (C) Cells were stained with Alcian blue. Extraction of stained cells was quantified by measuring the absorbance at 655 nm. (–, 200 μm).

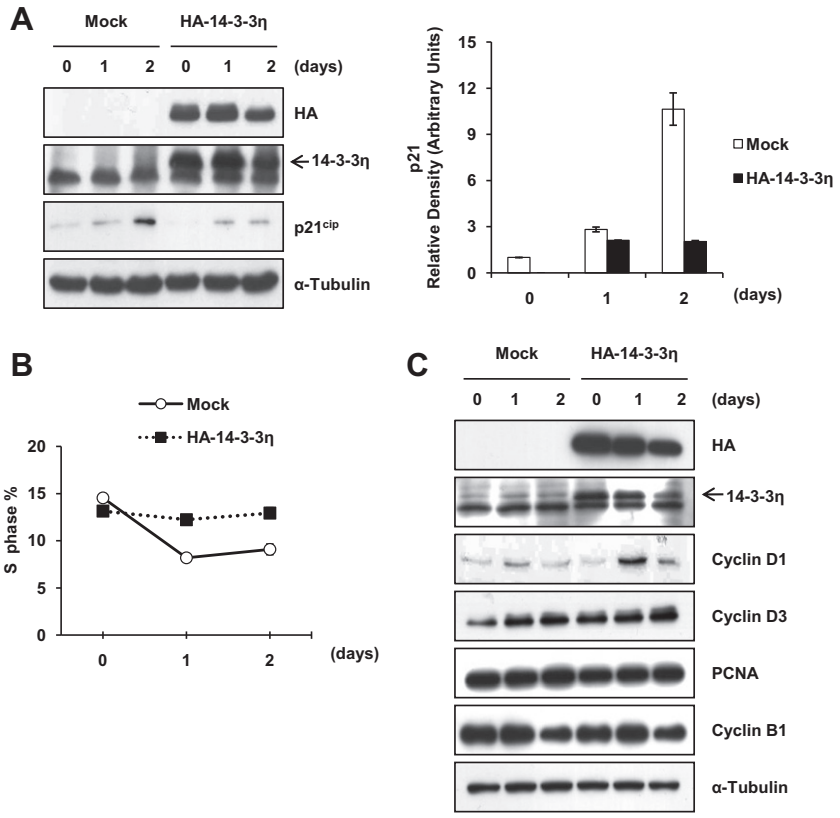

Fig. 4. Increased expression of 14-3-3η inhibits cell cycle arrest at G1 phase induced by insulin. ATDC5 cells were cultured in 60 mm plates at a density of 4×10^5 cells/well. Cells were then transfected with pcDNA3.1 control vector (mock) or expression vector containing HA-14-3-3η using lipofectamine 2000 (Invitrogen). After 24 h, medium was changed with or without insulin, after which cells were incubated for 2 days. (A) Expression levels of HA, 14-3-3η, and $p21^{cip}$ in mock and HA-14-3-3η transfected ATDC5 cells were determined by Western blot analysis (left panel). Relative density of $p21^{cip}$ was determined using a gel documentation system (right panel). (B) Percentage of cells in S phase of the cell cycle was determined by PI staining using flow cytometery. (C) Cell extracts from ATDC5 cells transfected with pcDNA3.1control vector (mock) or HA-14-3-3η were analyzed by immunoblotting with anti-HA, anti-14-3-3η, anti-cyclin D1, anit-cyclin D3, anti-PCNA, cyclin B1, or α-tubulin antibodies.

drogenesis. Further, Alcian blue staining showed that the formation of cartilage nodules was increased in the siRNA 14-3-3η-transfected ATDC5 cells (Fig. 3C). These indicate that expression of 14-3-3η suppresses chondrogenic differentiation.

3.4. Increased 14-3-3η inhibits cell cycle arrest of G1 phase induced by insulin

As shown in Fig. 1, 14-3-3η expression was increased when chondrogenesis was inhibited by TNF-α, and chondrogenesis was also inhibited by 14-3-3η overexpression. Thus, we next examined how 14-3-3η inhibits the chondrogenic differentiation of ATDC5 cells. The cdk inhibitor p21 is a well-known mediator of cell cycle arrest at G1 phase [26]. It has been shown that overexpression of p21 is essential for chondrocyte differentiation in ATDC5 cells, and thus cell cycle arrest at G1 phase is important to chondrogenesis [27,28]. Therefore, we determined the expression of p21 using ATDC5 cells transfected with HA-14-3-3η. While p21 expression was increased during chondrogenesis in mock control cells, it was not in ATDC5 cells transfected with 14-3-3η (Fig. 4A). Further, we found that the number of mock control cells in S phase between day 0 and day 1 of chondrogenesis was decreased by 43%. Thus, the cells were in G1 phase arrest. However, the number of 14-3-3η-transfected ATDC5 cells in S phase was decreased by only about 7% (Fig. 4B). These phenomena indicate that G1 phase arrest in chondrogenic differentiation was inhibited by overexpression of 14-3-3η. Cyclin D is synthesized during G1 phase and is required for the G1/S transition [29]. As shown in Fig. 4C, expression of both cyclin D1 and cyclin D3 was higher in 14-3-3η-transfected ATDC5 cells compared to mock control cells. However, expression of cyclin B1, which is involved in the G2/M transition, was unchanged in 14-3-3η-transfected ATDC5 cells. These results suggest that overexpression of 14-3-3η inhibits cell cycle arrest at G1 phase.

In this study, although the regulation mechanism is still unknown, our finding is the first study showing that 14-3-3η is involved in chondrogenesis. Thus, 14-3-3η is a key regulator in the growth impairment of children with joint inflammatory disease.

Acknowledgments

This research was supported by a National Research Foundation of Korea Grant funded by the Korean Government (Grant No. 2007-0053186). We thank Prof. J.S. Ko (Korea University) for kindly providing the plasmid expressing HA-14-3-3η.

References

[1] H. Fu, R.R. Subramanian, S.C. Masters, 14-3-3 proteins: structure, function, and regulation, Annu. Rev. Pharmacol. Toxicol. 40 (2000) 617–647.
[2] A. Aitken, D.B. Collinge, B.P. van Heusden, T. Isobe, P.H. Roseboom, G. Rosenfeld, J. Soll, 14-3-3 proteins: a highly conserved. Widespread family of eukaryotic proteins, Trends Biochem. Sci. 17 (1992) 498–501.
[3] A. Aitken, 14-3-3 and its possible role in co-ordinating multiple signalling pathways, Trends Cell Biol. 6 (1996) 341–347.
[4] C. Laronga, H.Y. Yang, C. Neal, M.H. Lee, Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression, J. Biol. Chem. 275 (2000) 23106–23112.
[5] M.J. van Hemert, H.Y. Steensma, G.P. van Heusden, 14-3-3 proteins: key regulators of cell division, signalling and apoptosis, BioEssays 23 (2001) 936–946.

[6] R.T. Kilani, W.P. Maksymowych, A. Aitken, G. Boire, Y. St-Pierre, Y. Li, A. Ghahary, Detection of high levels of 2 specific isoforms of 14-3-3 proteins in synovial fluid from patients with joint inflammation, J. Rheumatol. 34 (2007) 1650–1657.
[7] F. De Luca, Impaired growth plate chondrogenesis in children with chronic illnesses, Pediatr. Res. 59 (2006) 625–629.
[8] V.E. MacRae, C. Farquharson, S.F. Ahmed, The pathophysiology of the growth plate in juvenile idiopathic arthritis, Rheumatology 45 (2006) 11–19.
[9] H. Akiyama, J.P. Lyons, Y. Mori-Akiyama, X. Yang, R. Zhang, Z. Zhang, J.M. Deng, M.M. Taketo, T. Nakamura, R.R. Behringer, P.D. McCrea, B. De Crombrugghe, Interactions between Sox9 and beta-catenin control chondrocyte differentiation, Genes Dev. 18 (2004) 1072–1087.
[10] W. Bi, W. Huang, D.J. Whitworth, J.M. Deng, Z. Zhang, R.R. Behringer, B. de Crombrugghe, Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization, Proc. Natl. Acad. Sci. USA 98 (2001) 6698–6703.
[11] K. Martensson, D. Chrysis, L. Savendahl, Interleukin-1beta and TNF-alpha act in synergy to inhibit longitudinal growth in fetal rat metatarsal bones, J. Bone Miner. Res. 19 (2004) 1805–1812.
[12] S. Murakami, V. Lefebvre, B. de Crombrugghe, Potent inhibition of the master chondrogenic factor Sox9 gene by interleukin-1 and tumor necrosis factor-alpha, J. Biol. Chem. 275 (2000) 3687–3692.
[13] Z.P. Lum, B.E. Hakala, J.S. Mort, A.D. Recklies, Modulation of the catabolic effects of interleukin-1 beta on human articular chondrocytes by transforming growth factor-beta, J. Cell. Physiol. 166 (1996) 351–359.
[14] J.W. Harper, S.J. Elledge, Cdk inhibitors in development and cancer, Curr. Opin. Genet. Dev. 6 (1996) 56–64.
[15] J.A. Emons, R. Marino, O. Nilsson, K.M. Barnes, N. Even-Zohar, A.C. Andrade, N.A. Chatterjee, J.M. Wit, M. Karperien, J. Baron, The role of p27Kip1 in the regulation of growth plate chondrocyte proliferation in mice, Pediatr. Res. 60 (2006) 288–293.
[16] H. Hermeking, A. Benzinger, 14-3-3 proteins in cell cycle regulation, Semin. Cancer Biol. 16 (2006) 183–192.
[17] X.Q. Chen, S. Liu, L.Y. Qin, C.R. Wang, Y.W. Fung, A.C. Yu, Selective regulation of 14-3-3eta in primary culture of cerebral cortical neurons and astrocytes during development, J. Neurosci. Res. 79 (2005) 114–118.
[18] C. Shukunami, K. Ishizeki, T. Atsumi, Y. Ohta, F. Suzuki, Y. Hiraki, Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 in vitro, J. Bone Miner. Res. 12 (1997) 1174–1188.
[19] T. Atsumi, Y. Miwa, K. Kimata, Y. Ikawa, A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells, Cell Differ. Dev. 30 (1990) 109–116.
[20] A. Woods, G. Wang, H. Dupuis, Z. Shao, F. Beier, Rac1 signaling stimulates N-cadherin expression, mesenchymal condensation, and chondrogenesis, J. Biol. Chem. 282 (2007) 23500–23508.
[21] Y.S. Kim, S.W. Jang, H.J. Sung, H.J. Lee, I.S. Kim, D.S. Na, J. Ko, Role of 14-3-3 eta as a positive regulator of the glucocorticoid receptor transcriptional activation, Endocrinology 146 (2005) 3133–3140.
[22] K. Hidaka, T. Kanematsu, J.J. Caffrey, H. Takeuchi, S.B. Shears, M. Hirata, The importance to chondrocyte differentiation of changes in expression of the multiple inositol polyphosphate phosphatase, Exp. Cell Res. 290 (2003) 254–264.
[23] O. Nilsson, R. Marino, F. De Luca, M. Phillip, J. Baron, Endocrine regulation of the growth plate, Horm. Res. 64 (2005) 157–165.
[24] H. Ji, A. Pettit, K. Ohmura, A. Ortiz-Lopez, V. Duchatelle, C. Degott, E. Gravallese, D. Mathis, C. Benoist, Critical roles for interleukin 1 and tumor necrosis factor alpha in antibody-induced arthritis, J. Exp. Med. 196 (2002) 77–85.
[25] H. Saito, T. Kojima, M. Takahashi, W.C. Horne, R. Baron, T. Amagasa, K. Ohya, K. Aoki, A tumor necrosis factor receptor loop peptide mimic inhibits bone destruction to the same extent as anti-tumor necrosis factor monoclonal antibody in murine collagen-induced arthritis, Arthritis Rheum. 56 (2007) 1164–1174.
[26] K.F. Macleod, N. Sherry, G. Hannon, D. Beach, T. Tokino, K. Kinzler, B. Vogelstein, T. Jacks, P53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage, Genes Dev. 9 (1995) 935–944.
[27] M. Nakajima, Y. Negishi, H. Tanaka, K. Kawashima, P21(Cip-1/SDI-1/WAF-1) expression via the mitogen-activated protein kinase signaling pathway in insulin-induced chondrogenic differentiation of ATDC5 cells, Biochem. Biophys. Res. Commun. 320 (2004) 1069–1075.
[28] Y. Negishi, N. Ui, M. Nakajima, K. Kawashima, K. Maruyama, T. Takizawa, H. Endo, P21Cip-1/SDI-1/WAF-1 gene is involved in chondrogenic differentiation of ATDC5 cells in vitro, J. Biol. Chem. 276 (2001) 33249–33256.
[29] D. Resnitzky, M. Gossen, H. Bujard, S.I. Reed, Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system, Mol. Cell. Biol. 14 (1994) 1669–1679.