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Novel Role of miR-181a in Cartilage Metabolism

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ABSTRACT

Micro RNA (miRNA) is a small non-coding post-transcriptional RNA regulator that is involved in a variety of biological events. In order to specify the role of miRNAs in cartilage metabolism, we comparatively analyzed the expression profile of known miRNAs in chicken sternum chondrocytes representing early and late differentiation stages. Interestingly, none of the miRNAs displaying strong expression levels showed remarkable changes along with differentiation, suggesting their roles in maintaining the homeostasis rather than cytodifferentiation of chondrocytes. Among these miRNAs, miR-181a, which is known to play critical roles in a number of tissues, was selected and was further characterized. Human microarray analysis revealed remarkably stronger expression of miR-181a in human HCS-2/8 cells, which strongly maintained a chondrocytic phenotype, than in HeLa cells, indicating its significant role in chondrocytes. Indeed, subsequent investigation indicated that miR-181a repressed the expression of two genes involved in cartilage development. One was CCN family member 1 (CCN1), which promotes chondrogenesis; and the other, the gene encoding the core protein of aggrecan, a major cartilaginous proteoglycan, aggrecan. Based on these findings, negative feedback system via miR-181a to conserve the integrity of the cartilaginous phenotype may be proposed. J. Cell. Biochem. 114: 2094–2100, 2013.

KEY WORDS: CHONDROCYTE; miRNA; CCN1; CCN FAMILY; GENE REGULATION; HOMEOSTASIS

During this decade, critical roles of micro RNAs (miRNAs) in gene regulation in the human body have been uncovered by a vast number of studies. The miRNAs comprise a major class of non-coding RNA, and thousands have been identified to date. These miRNAs are transcribed as precursor forms referred to as pri-miRNAs and are processed into mature forms through serial digestion by nuclease complexes Drosha and Dicer [Kobayashi et al., 2008; Siomi and Siomi, 2010]. The mature miRNA is a small RNA duplex of 21–24 nucleotides in length. After being incorporated into the RNA-induced silencing complex (RISC), miRNA recognizes partially complementary nucleotide sequences in target mRNAs, which leads to translational repression or RNA degradation [Siomi and Siomi, 2010; Pasquinelli, 2012]. Since a single miRNA is predicted to control the fate of thousands of mRNAs, this post-transcriptional regulation is anticipated to be involved in almost all of the gene regulatory events occurring in the human body. Indeed, the critical contribution of a number of miRNAs to the development of a variety of physiological and pathological conditions has already been uncovered [Dews et al., 2006; Ohgawara et al., 2009; Pasquinelli, 2012], whereas the biological significance of other miRNAs still remains unclear.

The miR-181 family is one of the miRNAs, which functions have been extensively characterized. According to past studies, miR-181

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plays significant roles in early hematopoiesis and T-cell development [Chen et al., 2004; Li et al., 2007]. Also, miR-181 is known to regulate homeobox genes that conduct myoblast differentiation and lymphangiogenesis [Naguibneva et al., 2006; Kazenwadel et al., 2010]. More recent reports revealed the involvement of miR-181 in the inflammation process, T cell receptor sensitivity, cancer development, and mitochondrial function [Li et al., 2007; Fei et al., 2012; Ouyang et al., 2012; Zhao et al., 2012]. As such, this particular family of miRNAs may be considered as a critical regulator of tissue development and biological responses in multiple tissues. However, few findings on the miR-181 family have been presented in relation to cartilage biology.

We initiated the present study by performing a comprehensive microarray analysis to identify specific miRNAs that were associated with cartilage development and metabolism. Among the miRNAs that were highly expressed in chicken chondrocytes, we found a few miR-181 members. Our subsequent investigation revealed a novel role of miR-181a in chondrocytes, which finding significantly expands our knowledge about this miRNA as a regulator of multiple tissue development and maintenance.

MATERIALS AND METHODS

CELL CULTURE

Human cervical carcinoma HeLa, human chondrocytic HCS-2/8, and human breast cancer MDA-231 cell lines were cultured in Dulbecco's modified Eagle's minimum essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) [Takigawa et al., 1989; Ohgawara et al., 2011]. Chicken lower sternum (LS) and upper sternum (US) chondrocytes were isolated from the caudal and cephalic 1/3 portions, respectively, of the sternal cartilage of 16-day-old chicken embryos and maintained in high-glucose D-MEM supplemented with 10% FBS, as described previously [Enomoto-Iwamoto et al., 1998]. All cells were incubated at 37°C in humidified air containing 5% CO_2.

For evaluation of the effect of a recombinant CCN1 (rCCN1: Peprotech, Rocky Hill, NJ), HCS-2/8 cells were grown to confluence in regular medium; and the medium was then changed to D-MEM containing 0.5% FBS, and the cells were incubated for a further 24 h. Thereafter, rCCN1 was added to the cells at the desired concentration; and then total RNA was extracted and analyzed after 12 h, as described elsewhere.

RNA EXTRACTION AND MICROARRAY ANALYSIS

Total RNAs of HCS-2/8, HeLa, and MDA-231 cells, and of chicken chondrocytes, were isolated with Isogen® (Nippon Gene, Tokyo, Japan) by following the manufacturer's protocol. Subsequently, small RNAs were purified from these total RNAs and analyzed with FilgenArray *Gallus gallus* or mirVana™ miRNA Bioarray V2 (Ambion, Austin, TX) by Filgen, Inc. (Nagoya, Japan). For each miRNA, independent signals from 2 to 4 hybridizations were obtained and analyzed.

IN SILICO ANALYSES

Data obtained from microarray analyses were analyzed in silico by Microarray Data Analysis Tool (Filgen). For the prediction of miRNA targets in the *CCN1* 3′-UTR, several on-line devices were employed (Targetscan, http://www.targetscan.org/; miRanda, http://www.microrna.org/microrna/home.do; DIANA, http://diana.cslab.ece.ntua.gr/microT/) [Bartel, 2009].

SYNTHESIS AND APPLICATION OF miRNAs

Synthetic RNA oligonucleotides with the sequences 5′-AAC AUU CAA UGC UGU CGG UGA GU-3′ and 5′-ACC ACU GAC CGU UGA CUG UAC were prepared by B-Bridge International Inc (Cupertino, CA). These single-stranded RNAs were annealed to obtain the mature form of miR-181a duplexes. As a negative control, Silencer Negative Control siRNA (Ambion) was used. Control siRNA, luciferase (GL3; Cosmo Bio, Tokyo, Japan), was also employed as a positive control in reporter gene assays. Twenty-four hours before transfection, HCS2/8 or MDA-231 cells were seeded at 80–90% confluence, and miR-181a transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a concentration of 50 nM, according to the manufacturer's instruction. The medium was changed 24 h later, and the cells were incubated for a further 48 h for RNA isolation or for 72 h for protein extraction with 500 µl of a lysis buffer [Mukudai et al., 2005].

REVERSE TRANSCRIPTION AND QUANTITATIVE REAL-TIME PCR ANALYSIS

Reverse transcription was carried out by using avian myeloblastosis virus (AMV) reverse transcriptase with 500 ng of each total RNA according to the manufacturer's protocol (Takara, Ohtsu, Japan). After having been transcribed to cDNA, each mRNA level was quantitatively analyzed by the real-time PCR method with a LightCycler (Roche, Basel, Switzerland). Quantification was performed by intercalator methodology with a SYBR green real-time PCR master mix (Toyobo, Tokyo, Japan). The nucleotide sequences of the primers used for each mRNA were the following: 5′-AAC AAC TTC ATG GTC CCA GT-3′ (sense) and 5′-CTC AAA CAT CCA GCG TAA GT-3′ (antisense) for human *CCN1*; 5′-TCA AGG GCA TCC TGG GCT A C-3′ (sense) and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (antisense) for human *GAPDH*; 5′-TTC GGG CAG AAG AAG GAC-3′ (sense) and 5′-CGT GAG CTC CGC TTC TGT-3′ (antisense) for human *ACAN*; 5′-CAA TCC CGA CTG CAA GCT CAT-3′ (sense) and 5′- ACC TGC CAT CCA CGC AAG A-3′ (antisense) for chicken *CCN1*; 5′-AGG CTG TGG GGA AAG TCA-3′ (sense) and 5′-GAC AAC CTG GTC CTC TGT GTA T-3′ (antisense) for chicken *GAPDH*; 5′-CAC CAA CGA TAA TGC TTT C-3′ (sense) and 5′-ACT TAG CTC TGT ACG TCT TCA-3′ (antisense) for chicken *CCN2*; 5′-CCT GCC TGA CCT CTT TGC-3′ (sense) and 5′-TGG GGA GGA GGG CAA CAT-3′ (antisense) for chicken *ACAN*; and 5′- AGA AAG GAA TCC AGC CCA AT-3′ (sense) and 5′-ACA CCT GCC AGA TTG ATT CC-3′ (antisense) for chicken *COL2A1*.

WESTERN BLOTTING ANALYSIS

Extracted total proteins (20 µg) were heated at 95°C for 5 min in a sodium dodecyl sulfate (SDS) sample buffer with 2-mercaptoethanol, separated by 12% SDS–polyacrylamide gel electrophoresis and then transferred onto a polyvinylidenedifluoride (PVDF) membrane (GE Healthcare, Waukesha, WI). After having been blocked with 5% skim milk, the membrane was incubated for 24 h at 4°C with 500-fold-diluted rabbit polyclonal antibody against CCN1 (Santa Cruz

Biotechnology, Santa Cruz, CA) or 2,000-fold-diluted mouse monoclonal anti-actin antibody (SIGMA, St. Louis, MO). A secondary antibody at a 5,000-fold dilution, anti-rabbit or mouse IgG horseradish peroxidase (HRP) conjugate (GE Healthcare), was then applied. The blot was visualized by use of an enhanced chemiluminescence (ECL) system. Quantification of the signals was performed with Image J software (http://imagej.nih.gov/ij).

REPORTER GENE ASSAY

An miR-181a reporter construct, pGL3-181TS was constructed by inserting the predicted miR-181a target sequence in the *CCN1* mRNA 3′-UTR into a pGL3-L(+) vector [Kubota et al., 1999] at the multiple cloning sites located immediately downstream of the firefly luciferase gene. Namely, the sense and antisense single-stranded oligonucleotides containing the predicted target sequence were annealed and inserted into pGL3-L(+) that had been double-digested with *Xba*I and *Eco*RI. Integrity of the plasmids was confirmed by DNA sequencing. Twenty-four hours prior to transfection, HCS-2/8 cells were seeded into six-well tissue culture plates and then transfected with miR-181a or control siRNA, as described in another subsection. After an 8-h incubation, DNA transfection was conducted as described above with 500 ng of pGL3-181TS and 50 ng of phRL-TK (int-) (Promega); and the cells were harvested 48 h later with 500 µl of lysis buffer. Sequential measurement of firefly and Renilla luciferase activities was performed as described previously [Kubota et al., 1999].

STATISTICAL ANALYSIS

Unless otherwise specified, all of the evaluations were performed at least twice, yielding comparable results. Statistical significance of differences was examined by using Student's *t*-test.

RESULTS

RE-DISCOVERY OF miR-181a IN CHONDROCYTES BY COMPREHENSIVE ANALYSES

As an initial step of this study, we screened a microarray for chicken miRNAs with small RNAs extracted from chicken sternum chondrocytes to find out miRNAs that were highly expressed and played significant roles in cartilage metabolism. It is widely recognized that chondrocytes from the lower (LS) and upper (US) portions of sternum cartilage represent proliferative (immature) and prehypertrophic–hypertrophic (terminally differentiated) chondrocytes, respectively, in the growth plate [Mukudai et al., 2005]. As a result of the comparative analysis between these normal chondrocytes, 64 chicken miRNAs with guide-strand sequences were identified as those giving high signal intensities (>500 units) either in LS or US chondrocytes (Fig. 1A). Surprisingly, none of these miRNAs showed any remarkable increase or decrease in their expression levels during differentiation, with only one exception, miR-466. However, this miRNA is one of the miRNAs that is poorly conserved among vertebrates, and thus was not considered for further characterization.

Among the remaining 63 miRNAs, we were particularly interested in miR-181a and miR-181b, since the miR-181 family is one of the highly conserved mRNA families and is recognized to be a critical regulatory molecule in the other tissues, whereas little was known

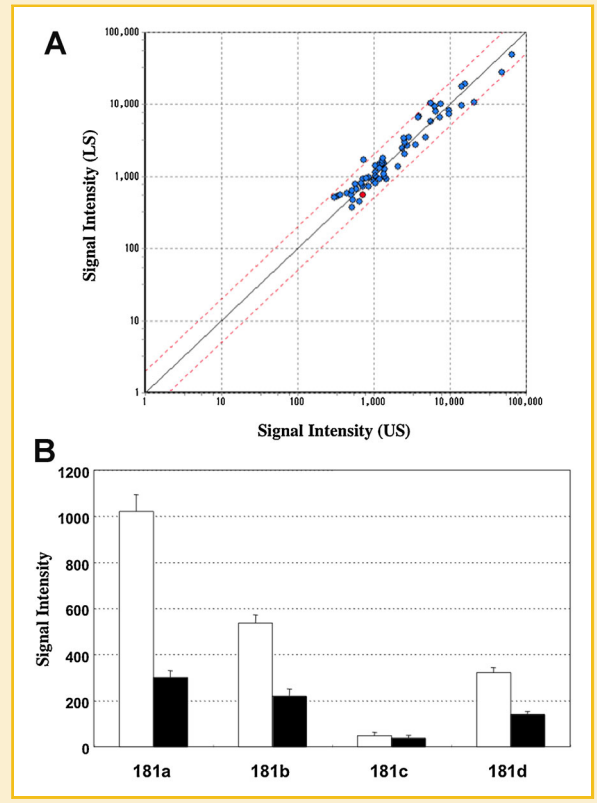

Fig. 1. Stable and specific expression of miR-181a in chondrocytes. A: Comparative display of the expression profiles of chicken miRNAs in LS and US chondrocytes. Signal intensities from the small RNAs in LS (ordinate) and US (abscissa) for each miRNA showing over 500 units on either microarray are plotted in log-scales. Dotted lines denote the positions of data with twofold difference between the two, thus representing the boundaries of significant and insignificant differences. Only one miRNA (miR-466) is plotted at a location outside of the boundary indicating significantly stronger expression in LS chondrocytes. Among these RNAs, miR-181a is indicated by a red dot. B: Expression profile of the miR-181 family in human chondrocytic HCS-2/8 cells, in comparison with that of it in HeLa cells. Signal intensities from four independent hybridizations in microarray analysis were subjected to computation of mean values and standard deviations, after subtraction of the background. Open and solid columns represent the data from HCS-2/8 and HeLa cells, respectively.

about this family in terms of cartilage biology. In addition to the initial screening, we also performed another microarray analysis, in order to specify human miRNAs that were specifically expressed in chondrocytes. For this experiment, small RNAs were prepared from two human cell lines, that is, HCS-2/8 and HeLa cells. HCS-2/8 cells, which had been established from a human chondrosarcoma, stably retain chondrocytic phenotype [Takigawa et al., 1989]. In contrast, HeLa cells, established from a cervical cancer, show no specific phenotype representing a particular tissue, although chondrocytic differentiation can be induced to a limited degree by the overexpression of multiple transcription factors [Ikeda et al., 2004]. The results of comparative analysis of the expression of miRNAs between these two cell lines revealed 22 human miRNAs that displayed more

than twice higher expression levels in HCS-2/8 cells than in HeLa ones. To our surprise, 3 out of the 22 were from the miR-181 family, in which miR-181a presented the highest expression level among the members (Fig. 1B). As such, miR-181a was selected for subsequent investigation.

PREDICTION OF miR-181a TARGET GENES IN SILICO

Since any miRNA supposedly regulates thousands of mRNAs, we initially selected putative mRNAs that could be targeted by miR-181a by performing in silico analysis. Nowadays, several on-line devices based on different algorithms are available for the prediction of miRNA targets [Bartel, 2009]. Among them, Targetscan, DIANA, and miRanda were employed to specify the candidates in this study. The results of such analysis collectively identified two putative targets that were critically involved in chondrogenesis, one being *CCN1*, and the other, *ACAN*. The CCN1 protein is known to promote chondrogenesis [Wong et al., 1997; Chen and Lau, 2009]; whereas *ACAN* encodes the core protein of aggrecan, which is the major proteoglycan found specifically in cartilage. Indeed, two putative sequences recognized by miR-181a, which share striking homology with those in other targets that had been already experimentally proven to be responsive to miR-181a, were predicted in their 3′-UTRs (Fig. 2).

REGULATION OF CCN1 EXPRESSION BY miR-181a

In general, miRNAs regulate gene expression via two mechanisms that are related to each other. One is the degradation of target mRNAs, and the other is the repression of protein translation [Pasquinelli, 2012]. Firstly, we evaluated the former mode of regulation by miR-181a by performing mRNA analysis with two human cell lines, HCS-2/8 and MDA231, both of which are known to express *CCN1* [Moritani et al., 2005; Ohgawara et al., 2011]. The introduction of synthetic mature miR-181a resulted in a significant reduction in the steady-state CCN1 mRNA level in human breast cancer MDA-231 cells (Fig. 3A). However, no such effect was observed in chondrocytic HCS-2/8 cells (Fig. 3A). Next, production of CCN1 protein was analyzed by Western blotting of cell lysates after the introduction of exogenous miR-181a. CCN1 production was repressed in both cell lines by exogenous miR-181a (Fig. 3B,C). These results indicate the cell-type dependence of the miR-181a action, implying that the inhibition of protein translation is more important than mRNA degradation, especially in chondrocytes.

Functional targets	*Prox1*	5′AACUCUUUUUGAAUGUA3′
	CDX2	5′UCUCUGGGCUGAAUGUA3′
	GATA6	5′UUUUUAUAAUGAAUGUA3′
	NLK	5′CUUGAAACUUGAAUGUC3′
	Hox-A11	5′UAAAAUAUUUGAAUGUA3′
Predicted targets	*CCN1*	5′UUUUAAGACUGAAUGUU3′
	ACAN	5′AACCGCAGCUGAAUGUA3′

Fig. 2. Nucleotide sequence alignment of the miR-181a targets in the 3′-UTR of corresponding mRNAs. The five RNA sequences shown in the upper part of the figure had been experimentally proven to be functional in previous studies [Naguibneva et al., 2006; Ji et al., 2009; Kazenwadel et al., 2010], whereas the bottom two sequences were predicted by multiple in silico analyses in this present study. Shaded area indicates the seed sequences perfectly conserved among all of the mRNAs.

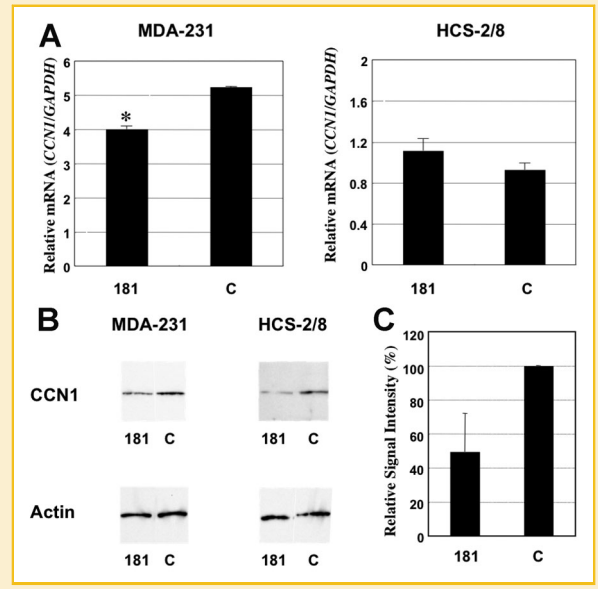

Fig. 3. Effect of exogenous miR-181a on endogenous CCN1 gene expression in human chondrocytic and breast cancer cells. A: Effect on steady-state *CCN1* mRNA level. Synthetic miR-181a (181) or control (C) siRNA duplexes were used to transfect MDA-231 and HCS-2/8 cells, and steady-state *CCN1* mRNA levels were evaluated by real-time RT-PCR analysis 48 h later. Relative *CCN1* mRNA levels were computed by standardizing the data against those of *GAPDH*. Mean values are presented with error bars indicating SD values. An asterisk (*) indicates a value significantly lower than the control one at $P < 0.01$. The *CCN1* mRNA level was lowered in the MDA-231 cells, but unaffected in chondrocytic HCS-2/8 cells, by miR-181a. B: Effect on CCN1 protein production. Transfection of the indicated cells with miR-181a was performed as described above, and CCN1 protein and actin (control) levels in the cell lysate were evaluated by Western blotting. CCN1 protein production was repressed in both cells. C: Quantitative analysis of independent sets of the Western blotting data with HCS-2/8 cells. Data are standardized by those of actin and represented as percentages against the control. Error bar denotes standard deviation of the relevant data.

PUTATIVE miR-181a TARGET(S) IN THE HUMAN CCN1 GENE

Since an miR-181 target was predicted in silico, we examined whether or not the predicted target sequence in *CCN1* mRNA 3′-UTR was a functional target for miR-181a by performing a reporter gene assay. The predicted target sequence was synthesized, inserted into a downstream site of the luciferase gene driven by an SV40 promoter to obtain the reporter construct designated as pGL3-181TS (Fig. 4A). Co-transfection of HCS-2/8 cells with a chemically synthesized mature miR-181a duplex and pGL3-181TS, however, resulted in only a mild repression of luciferase gene expression, compared with the results obtained with the control double-stranded RNA (Fig. 4B). Under the same experimental conditions, an siRNA against firefly luciferase distinctly repressed the expression of the marker gene (Fig. 4C). These results suggest that mRNA degradation is not the primary route, by which miR-181a regulates CCN1, and also indicate a minor role of the putative target predicted therein.

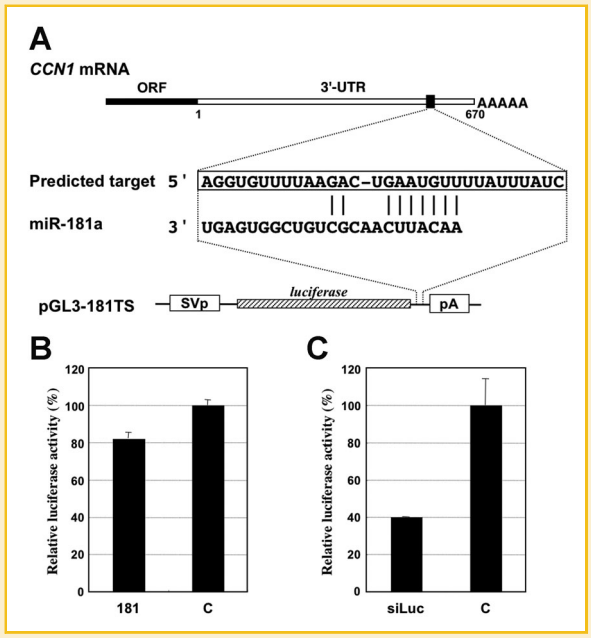

Fig. 4. Functional evaluation of the predicted miR-181a target sequence in the CCN1 3′-UTR. A: Structure of the plasmid used to evaluate the function of the predicted miR-181a target. The predicted target sequence located at the solid small box in the *CCN1* 3′-UTR was synthesized and inserted into the parental reporter plasmid to yield pGL3-181TS. The nucleotide sequence of the inserted DNA is illustrated in the middle of the panel, together with that of miR-181a. Lines between the sequences denote the complementary nucleotides. B: Relative luciferase activities from the plasmid illustrated in A in HCS-2/8 cells in the presence or absence of exogenous miR-181a. The cells were transfected with miR-181a or control siRNA duplex. Eight hours later, the cells were further transfected with the reporter plasmid along with an internal control, phR-TK (int-), and were harvested for the luciferase assay after 48 h of incubation. Data are presented as relative values against those from the control samples. C: Control experiments under the same condition as that for panel B with a parental plasmid and an siRNA against the luciferase mRNA (siLuc).

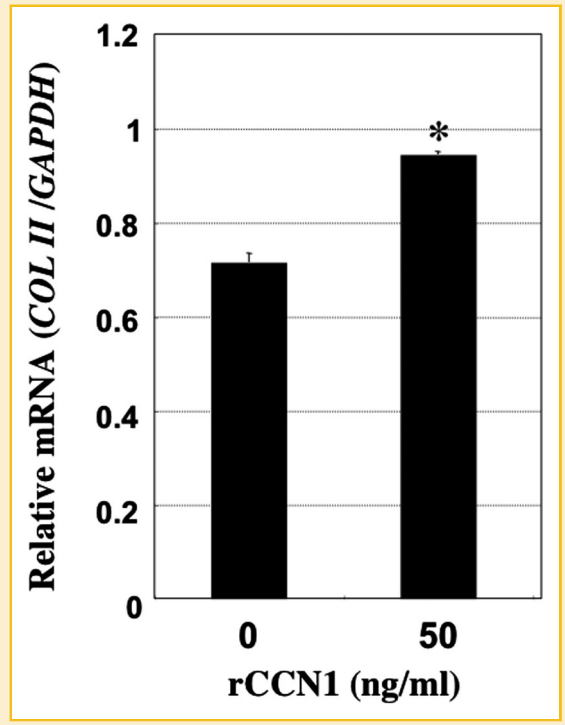

Fig. 5. Effect of rCCN1 on the expression of type II collagen gene in HCS-2/8 cells. Confluent HCS-2/8 cells were treated by the indicated concentration of rCCN1 for 24 h and total RNA was extracted for the evaluation by real-time RT-PCR analysis. The expression levels were standardized against those of *GAPDH*. Results are shown with error bars indicating standard deviations. Asterisk (*) represents significant difference at $P < 0.05$ against the control without rCCN1.

EFFECT OF A RECOMBINANT CCN1 ON CHONDROCYTE MARKER GENE EXPRESSION

In a previous study, CCN1 was described to promote chondrogenesis in vitro [Wong et al., 1997]. If so, miR-181a may be regulating chondrocyte metabolism indirectly via CCN1. To confirm the significance of this biological pathway, we evaluated the effect of a rCCN1 on the metabolism of HCS-2/8 cells. Type II collagen is the major collagen in cartilage, while aggrecan is the major proteoglycan therein. As shown in Figure 5, the addition of exogenous rCCN1 significantly promoted the expression of the type II collagen gene. We could detect no significant enhancement in the aggrecan core protein gene expression with rCCN1, probably because of highest basal level of constitutive *ACAN* expression in this cell line (data not shown).

REGULATION OF CHONDROCYTE MARKER GENES BY miR-181a IN DIFFERENTIATED CHONDROCYTES

Although HCS-2/8 is an established cell line that retains chondrocytic phenotype at a high degree, this tumor-derived cell line does not follow chondrocytic differentiation pathway toward hypertrophy, staying stably at a mature stage. Therefore, in order to confirm the biological effects of miR-181a on chondrocytes, we examined the effects of exogenous miR-181a on the expression of chondrocytic marker genes in normal US chondrocytes representing the prehypertrophic/hypertrophic phenotype (Fig. 6). As a result, the type II collagen gene showed a tendency toward decreased expression after transfection with miR-181a although the change was not statistically significant. It should be noted that *ACAN* was strongly repressed by miR-181a, which finding is consistent with the in silico prediction results. In addition, the expression of the CCN2 gene, a marker of early hypertrophic differentiation, showed no significant change, indicating minimal effect on the late differentiation of chondrocytes. Taken together with the results on the CCN1 gene expression, these results indicate the functionality of miR-181a as a regulator of chondrocytic metabolism.

DISCUSSION

In the present study, we for the first time uncovered a functional aspect of miR-181a as a regulator of chondrocyte metabolism. Previous studies indicated that miR-181a is a fundamental regulator of the development of various biosystems. According to one such past study, this miRNA is highly expressed in the thymus and represses multiple phosphatase genes in the T-cells, leading to the activation of T-cell receptor signaling [Li et al., 2007]. Since we confirmed

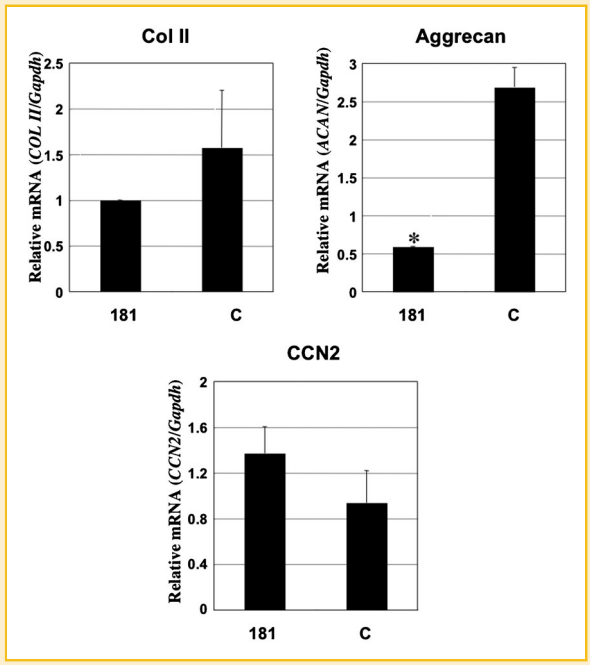

Fig. 6. Effect of exogenous miR-181a on the chondrocyte marker genes in chicken US chondrocytes. Chicken US cells were transfected with miR-181a (181) or control (C) siRNA duplexes; and the mRNA levels of the type II collagen (*COL II*), aggrecan core protein (*ACAN*), and CCN2 (*CCN2*) genes were evaluated by quantitative real-time RT-PCR. Relative mRNA levels standardized against those of *GAPDH* are presented with error bars indicating SD values. The asterisk (*) indicates a value significantly different between the two groups at $P < 0.05$.

remarkable expression of miR-181a in chondrocytes as well, we suspected such a multiple-targeted regulation by miR-181a in these cells. Then, as a result of combinatory in silico analysis, two putative targets, *CCN1* and *ACAN*, both of which are closely related to chondrogenesis, were identified. Consistent with this prediction, evaluation of the expression of endogenous *CCN1* and *ACAN* genes with and without exogenous miR-181a in fact showed clear repressive effects of this miRNA on these molecular targets. CCN1 is a representative member of the CCN family of proteins [Perbal and Takigawa, 2005; Kubota and Takigawa, 2007, 2011; Chen and Lau, 2009] and plays critical roles in chondrogenesis, cardiovascular development, and angiogenesis [Wong et al., 1997; Mo et al., 2002; Moritani et al., 2005; Chen and Lau, 2009], whereas aggrecan is an indispensable proteoglycan that furnishes cartilaginous tissues with an important physical property responding in mechanical stress loading. Therefore, regulation of multiple target genes critically involved in tissue development was found to be exerted by miR-181a in cartilage as well.

One of the issues remaining to be clarified is the mechanism by which *CCN1* and *ACAN* are regulated by miR-181a. It is widely recognized that miRNA target sequences are mostly located in the 3′-UTR of an mRNA. As was shown in Figure 2, putative miR-181a targets that were highly homologous to known functional targets were indeed predicted to be present in the 3′-UTRs of *CCN1* and *ACAN*. Based on these data, we evaluated the responsiveness of these sequences to miR-181a by using a conventional reporter gene assay system. Unexpectedly, we observed only a modest repression by miR-181a of the reporter gene with the target predicted in the *CCN1* 3′-UTR. We also performed the same assay using a reporter construct with the target predicted to be present in *ACAN*; nevertheless, no response to miR-181a was found (data not shown). With any other prediction tool utilized, no other target sequences were predicted in the UTR of either *CCN1* or *ACAN*. One possible explanation for these findings is that, although it has been widely recognized that most miRNA targets are located in the 3′-UTR, functional miR-181a targets may also exist elsewhere in *CCN1* and *ACAN* mRNAs [Pasquinelli, 2012]. Alternatively, the predicted targets may be necessary, but not sufficient to confer full responsiveness to miR-181a; thus other *cis*-elements outside of the 3′-UTRs may be required in addition. Another interpretation of these results is that *CCN1* and *ACAN* may be indirectly regulated by miR-181a via some unknown target gene(s). This hypothesis appears more likely in the case of *ACAN*, as the effect of exogenous miR-181a on normal chondrocytes appeared too strong to be regarded as a direct effect of a single miRNA. It should be also noted that exogenous miR-181a did not significantly repress *ACAN* expression in HCS-2/8 cells (data not shown), which constitutively express *ACAN* without moving backward through the differentiation pathway [Takigawa et al., 1989]. Considering these data together, it may be more feasible to regard the effect of miR-181a on *ACAN* mostly as the outcome of indirect effects of this particular miRNA. Since CCN1 is one of the molecules that promote chondrogenesis (Fig. 5), the negative effect of miR-181a on CCN1 and other unknown targets may cause the reduction in *ACAN* expression in normal chondrocytes. As such, a portion of the strong effect of miR-181a against *ACAN* may be ascribed to its indirect effect via *CCN1* repression. These points are currently under further investigation.

In spite of its repressive role in cartilage metabolism, miR-181a is expressed constantly until terminal differentiation stages. Considering that chondrocytes are actively producing cartilaginous extracellular matrix (ECM) components even in the presence of miR-181s, our data suggest that miR-181a acted as a negative feedback mediator to maintain the homeostasis in cartilage metabolism. As also stated above, miR-181a induced during chondrogenesis down-regulates chondrogenesis via CCN1, as well as ECM construction via aggrecan, thus forming a dual-feedback system for the maintenance of cartilage homeostasis. The possible contribution of the two other miR-181 members, miR-181b and d, should also be noted here.

Endochondral ossification is the central biological process for skeletal formation and is maintained by a number of regulatory molecules. Our present study further emphasizes the critical importance of miRNA-mediated post-transcriptional gene regulation in skeletal development. Investigation of upstream factors that regulate miR-181a expression may shed light on the complex molecular network regulating cartilage metabolism, in which the miR-181a system described here is involved as one of the players.

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