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Autoimmune regulator, *Aire*, is a novel regulator of chondrocyte differentiation

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ABSTRACT

Chondrocyte differentiation is controlled by various regulators, such as Sox9 and Runx2, but the process is complex. To further understand the precise underlying molecular mechanisms of chondrocyte differentiation, we aimed to identify a novel regulatory factor of chondrocyte differentiation using gene expression profiles of micromass-cultured chondrocytes at different differentiation stages. From the results of microarray analysis, the autoimmune regulator, Aire, was identified as a novel regulator. Aire stable knockdown cells, and primary cultured chondrocytes obtained from *Aire*$^{-/-}$ mice, showed reduced mRNA expression levels of chondrocyte-related genes. Over-expression of Aire induced the early stages of chondrocyte differentiation by facilitating expression of *Bmp2*. A ChIP assay revealed that Aire was recruited on an Airebinding site (T box) in the *Bmp2* promoter region in the early stages of chondrocyte differentiation and histone methylation was modified. These results suggest that Aire can facilitate early chondrocyte differentiation by expression of *Bmp2* through altering the histone modification status of the promoter region of *Bmp2*.

Taken together, Aire might play a role as an active regulator of chondrocyte differentiation, which leads to new insights into the regulatory mechanisms of chondrocyte differentiation.

1. Introduction

Chondrocyte differentiation is a process that establishes skeletal morphology during embryonic development as well as longitudinal growth after birth. Chondrocytes are differentiated from mesenchymal stem cells (MSCs), with subsequent differentiation into proliferative chondrocytes and then into hypertrophic chondrocyte [1,2]. This sequentialdifferentiation is regulated by various factors such as SRY-box containing gene 9 (Sox9), Indian hedgehog (Ihh), and parathyroid hormone-related protein (PTHrP) [1]. In early chondrocyte differentiation, Sox9, which is a crucial transcription factor mediated by Ihh/PTHrP and bone morphogenetic protein (BMP) signaling [3], regulates cartilage formation via upregulation of chondrocyte-specific genes such as *Col2a1* by cooperating with various proteins [4–6]. During the maturation stage, the expression of Sox9 can be down-regulated by RelA (a member of the NF-κB family). Subsequently, runt-related transcription factor 2 (Runx2) can induce differentiation into hypertrophic chondrocytes, which have high expression of *Col10a1* [7].

The mutation of the gene locus of these known factors can cause severe metaphyseal and/or epiphyseal dysplasias and chondrodysplasia [8–10]. However, the genes responsible for various osteochondrodysplasias and chondrocyte-related diseases remain elusive. It is necessary to understand the molecular mechanisms underlying chondrocyte differentiation for investigation into novel approaches to treat cartilage-related diseases. Consequently, identification of novel factors driving the process of chondrocyte differentiation, especially those related to epigenetic regulation, should be considered.

Epigenetic regulation is a mechanism that can control chromatin dynamics followed by transcriptional activation and repression [11]. Epigenetic regulations are mediated through reversible chemicalmodifications on DNA and histone proteins, and their recognition by various enzymes and nuclear proteins [12]. It has been reported that these epigenetic regulators play a significant role *in vivo* as well as *in vitro* [13–15]. However, epigenetic regulations in chondrocyte differentiation are still largely unknown. Thus, the purpose of this study was to identify novel transcription and/or epigenetic factors in chondrocyte differentiation in order to investigate the underlying molecular

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mechanisms using an *in vitro* micromass culture system and gene expression microarrays.

2. Materials and methods

2.1. Animals

C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and *Aire*$^{-/-}$ mice were kindly provided from Dr. Mitsuru Matsumoto at Tokushima University, Japan [16]. All animals were maintained according to a protocol approved by the Animal Care and Use Committee of the University of Tokyo.

2.2. Chondrocytogenic cell culture

C3H10T1/2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS, Nichirei Biosciences, Tokyo, Japan) and antibiotic–antimycotic (Gibco, USA) at 37 °C in an atmosphere of 5% CO_2. To induce chondrocyte differentiation, micromass-culturedC3H10T1/2 cells were treated with 100 ng/ml of recombinant human BMP2 (Osteopharma, Osaka, Japan) as previously reported [17]. Medium was changed every 2 days.

The early stage chondrogenic cell line, ATDC5, was maintained in DMEM/F12 (Gibco) with 5% FBS and antibiotic–antimycotic.

Primary chondrocytes were isolated from articular cartilage of postpartum mice at day 6 as previously reported [18]. The isolated chondrocytes were cultured at a density of 10^4 cells/well in a 24-well plate with 1.5 ml/well of DMEM (high glucose) with antibiotic–antimycotic and 10% FBS under sterile conditions in an atmosphere of 5% CO_2 at 37 °C. The culture medium was changed every 2 days. Cells were harvested at day 7 for RT-PCR.

2.3. Gene expression microarray analysis

Micromass-cultured cells were harvested at days 0, 5, 9 and 15, and gene expression microarrays were performed as described previously [19,20]. A heat map was generated with Multi Experiment Viewer software. Data were clustered and analyzed with DAVID bioinformatics database (http://david.abcc.ncifcrf.gov/) [21].

2.4. Real-time RT-PCR

Total RNA was extracted with Trizol (Invitrogen, USA) and was subsequently treated with DNase I (Takara BioInc., Otsu, Japan). First-strand cDNA was synthesized from total RNA using PrimeScript RT Master Mix (Takara Bio Inc.) and subjected to real-time RT-PCR using SYBR Premix Ex Tag II (Takara Bio Inc.) with Thermal Cycler Dice (Takara Bio Inc.) according to the manufacturer's instructions. Primers were purchased from Operon Biotechnologies (Tokyo, Japan) and the sequences of the primer sets are shown in Supplementary Table 1.

2.5. Immunohistochemistry (IHC)

Immunohistochemistry was performed as previously described [19]. Briefly, paraffin sections of decalcified tibiae of 9-week-old C57BL/6 mice were incubated overnight with primary antibodies (anti-Aire, LS-C29969, Lifespan, 1:1000 diluted by 2% goat serum in PBST) and then treated with a biotinylated secondary antibody (Vector Laboratories) and an avidin–biotin peroxidase complex (ABC Vectastain Kit, Vector). Diaminobenzidine tetrahydrochloride (Sigma) was the chromogen. Negative controls were prepared by omitting the primary antibody and replacing it with non-immune serum at the same dilution.

2.6. Establishment of Aire stable knockdown cell line

Knockdown experiments were performed as previously described [13]. Briefly, oligonucleotide sequences for short hairpin RNA (shRNA) targeting *Aire*, *Sox9* and *LacZ* were designed via BLOCK-iT™ RNAi designer (Invitrogen) and cloned into the pSUPER.reto.puro vector. Each vector was transfected into the Platinum-E cell line with lipofectamine2000 (Invitrogen). After 2 days,retrovirus was produced and infected into C3H10T1/2 cells with 10 μg/ml polybrene. 24 h later, cells were passaged and treated with puromycin (5 μg/ml) to select the infected cells. Culture medium was changed every 2 days. After 7 days, the stable knockdown cells were produced and the efficiency of knockdown was determined by real-time RT-PCR.

2.7. Luciferase reporter assay

A reporter plasmid was constructed using pGL3 (Promega) and 2 kb upstream from the transcriptional start site of the *Bmp2* gene. A reporter assay for the detection of transcriptional activity was performed with the Dual-Luciferase Reporter assay system (Promega). Approximately 80% confluent ATDC5 cells were transfected with *Bmp2* promoter–luciferase reporter plasmids with expression vectors of Aire. For evaluating transfection efficiency, the renilla-luciferase gene derived from the CMV promoter was used. Transfection was performed with Superfect transfection reagent (QIAGEN) according to the manufacturer's instructions. A dual luciferase assay was performed according to the manufacturer's instructions. In each experiment, firefly luciferase activity was normalized to renilla luciferase activity.

2.8. Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed as described [13,22] with anti-Aire (ab13573, Abcam), anti-H3K4me2 (ab7766, Abcam) and normal goat or rabbit IgG as a control. Primer sets used for PCR were as follows: Aire binding T box site forward primer; 5′-CAAAACAGAAGC GTTTGCTCAC-3′, reverse primer;5′-TGGCCTCTGAGTTCCCTCATT-3′ and negative control site (about -5000 bp) forward primer; 5′-TCACTGGTACTTATGGCTGTGATG-3′, reverse primer; 5′-TCTGTGTT CCCTGCTCTGCT-3′.

2.9. Statistical analysis

We used one-way analysis of variance (ANOVA) to initially determine whether an overall statistically significant change existed before using Tukey's *post hoc* test and a two-tailed Student's *t* test to analyze the differences between two groups. For all graphs, data are represented as the mean ± S.D. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Identification of the novel transcriptional factor, Aire, in chondrocyte differentiation

To identify novel transcriptional and/or epigenetic regulators in chondrocyte differentiation, micromass-cultured C3H10T1/2 cells were analyzed to establish chondrocyte differentiation *in vitro.* RT-PCR showed that *Col2a1* was highly expressed at day 5 and subsequently decreased, whereas the expression peak of *Col10a1* was detected at day 9 compared with other time points (Fig. 1A). This suggested that the micromass-cultured cells at days 0, 5 and 9 could be considered as MSCs, proliferative chondrocytes and hypertrophic chondrocytes, respectively.

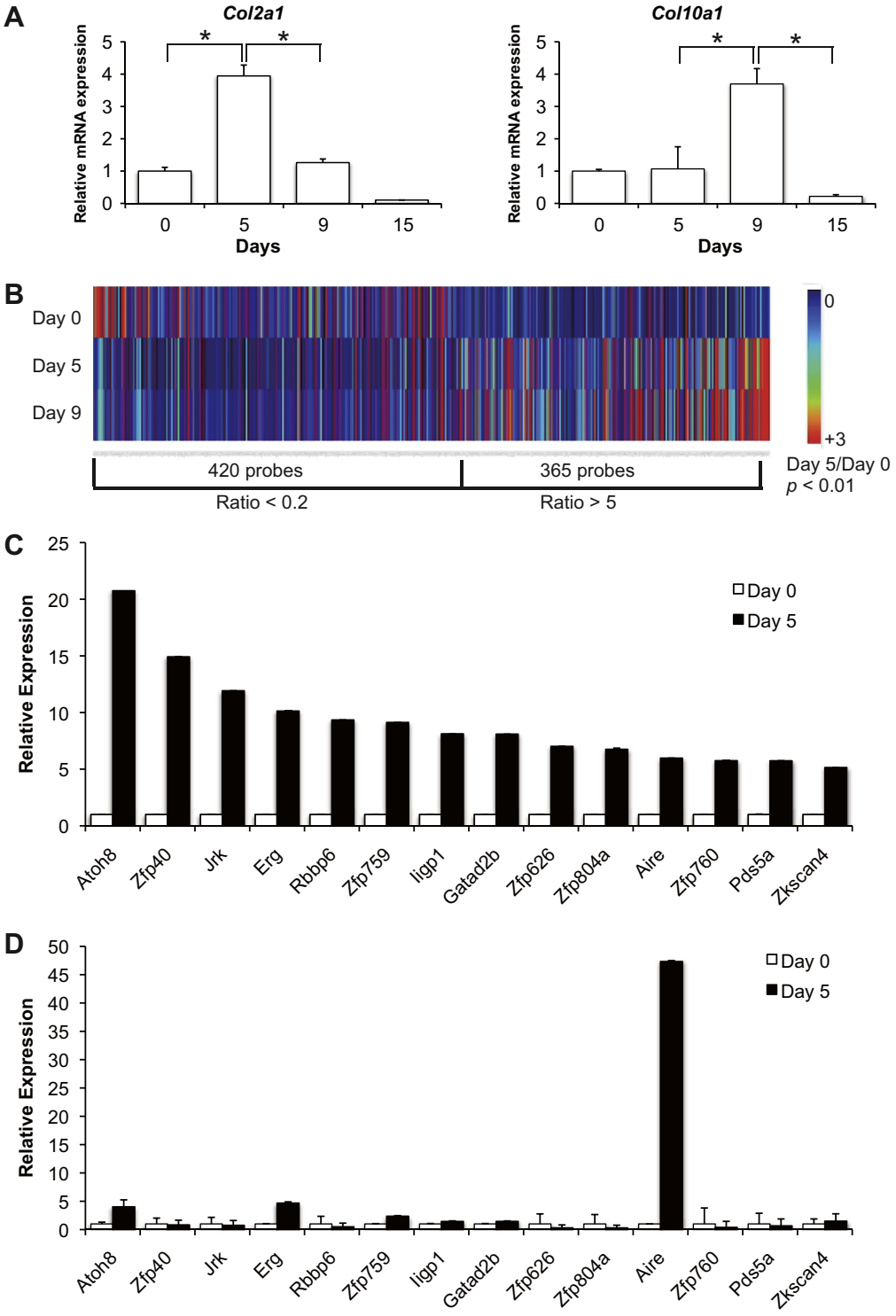

Fig. 1. Aire was identified as a novel transcriptional factor in chondrocyte differentiation. (A) Expression levels of *Col2a1* and *Col10a1* of micromass-cultured C3H10T1/2 cells treated with BMP2 for 0, 5, 9 and 15 days as determined by real-time RT-PCR and normalized to *Gapdh*. (B) A heat map of differentially expressed genes comparing day 0 and day 5. Genes with more than a 5-fold increase or 0.2-fold decrease as generated via Multi Experiment viewer software. (C) Expression levels of the genes categorized as "transcription" in the microarray. (D) Validation of microarray data by real-time RT-PCR and normalized to *Gapdh*. Data are presented as mean ± S.D. * indicates $p < 0.05$.

Based on the established pattern of chondrocyte differentiation, data from gene expression microarray showed a 5-fold significant difference of 785 genes (up-regulated: 365, down-regulated: 420) that were differentially expressed between day 0 and day 5 (Fig. 1B). All of them were clustered and analyzed based on the keyword "transcriptional factors" via the DAVID bioinformatics database and 14 factors were identified as possible candidates (Fig. 1C). After validation using real-time RT-PCR, the expression levels of *Aire* at day 5 were confirmed as the most significant increase compared to that at day 0 (Fig. 1D). Thus, Aire was suggested to be a novel transcription regulatory factor in chondrocyte differentiation.

3.2. Aire may activate the early stages of chondrocytedifferentiation

To determine the expression of Aire during chondrocyte differentiation *in vitro* and *in vivo*, real-time RT-PCR and IHC was performed. *Aire* was notably expressed after day 5 of chondrocyte differentiation (Fig. 2A) and Aire positively-stained cells were predominantly localized in the proliferative zone as well as the hypertrophic zone, but not in the resting zone of mouse tibiae (Fig. 2B). Therefore, the change in expression profile of Aire occurred in the early stages of chondrocyte differentiation.

Next, to determine whether Aire affected chondrocyte differentiation, we knocked down *Aire* in micromass-cultured C3H10T1/2 cells and harvested cells at days 0, 5 and 9 after BMP2 treatment. The knockdown efficiency of Aire-shRNA (sh*Aire*) was confirmed by RT-PCR compared with LacZ-shRNA (sh*LacZ*)-infected cells (Fig. 2C). The expression of chondrocyte differentiation marker genes (*Col2a1* and *Col10a1*) was decreased by *Aire* knockdown (Fig. 2C). These data suggested that Aire might be regarded as an activator of chondrocyte differentiation.

3.3. Aire may up-regulate Bmp2 expression

Our results suggested that Aire may up-regulate chondrocyte-related genes and promote the early stages of chondrocyte differentiation. However, the target genes of Aire in chondrocyte differentiation are unknown. Ithas been documented that the expression of *Bmp2*, the well-known chondrocyte differentiation inducer, was repressed in *Aire*$^{-/-}$ thymic epithelial cells [10]. Thus, it was hypothesized that Aire might activate chondrocyte-related genes via regulation of *Bmp2* expression.

As micromass-cultured C3H10T1/2 cells are unable to differentiate into chondrocytes without the induction of BMP2 we knocked down *Aire* in ATDC5 cells to determine the effect of Aire on endogenous *Bmp2*. At day 2, ATDC5 cells were harvested and the RNA was extracted to analyze the mRNA expression of *Bmp2*. *Bmp2* expression was significantly decreased in *Aire* knockdown ATDC5cells (Fig. 3A). In addition, this result was confirmed in primary cultured chondrocytes obtained from *Aire*$^{-/-}$ mice and wild-type littermates (Fig. 3B). These results indicated that Aire might regulate expression of *Bmp2* in the early stages of chondrocyte differentiation.

Furthermore, to determine whether Aire could directly regulate transcription of *Bmp2* in early stages of chondrocyte differentiation, transcriptional activity of Aire was examined by a luciferase reporter assay using the *Bmp2* promoter. The results showed that Aire could significantly increase *Bmp2* promoter activity in ATDC5 cells (Fig. 3C). In addition, overexpression of Aire could induce *Bmp2* expression as well as *Col2a1* and *Sox9* expression in C3H10T1/2 cells without BMP2 treatment (Fig. 3D). Taken together, these results indicate that Aire may facilitate chondrocyte differentiation via transcriptional activation of *Bmp2* expression.

3.4. Aire bound to the Bmp2 promoter with alteration of H3K4me2 modification

Aire consensus-binding motifs have been identified as the ATTGGTTA (G box) and TTATTA (T box) [23]. The T box motif exists in the *Bmp2* promoter (Fig.4A: −1503 to −1497 bp). To determine whether Aire bound to the endogenous *Bmp2* promoter, we performed a ChIP-qPCR assay using shAire C3H10T1/2 cells treated with BMP2 for 1 or 3 days. Aire binding was confirmed at the T box site, but not at the negative control site, in the *Bmp2* promoter (Fig. 4B). As expected, the recruitment of Aire was significantly decreased in shAire cells. To further understand the molecular mechanisms of Aire on the *Bmp2* promoter in chondrocyte differentiation, we investigated histone H3K4 modification, especially

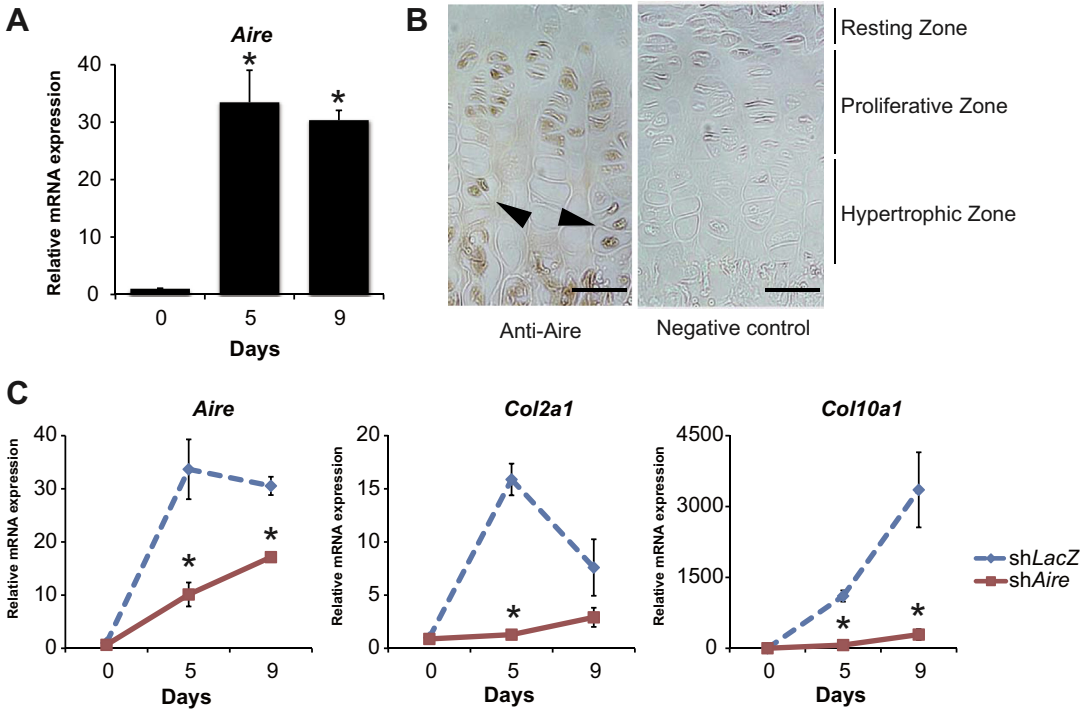

Fig. 2. Knockdown of *Aire* decreased the expression of chondrocyte-related genes. (A) Expression levels of *Aire* of micromass-cultured C3H10T1/2 cells treated with BMP2 for 0, 5 and 9 days as determined by real-time RT-PCR and normalized to *Gapdh*. (B) Immunohistochemistry of Aire in the proximal tibial growth plate. Immuno-positive cells stained in brown were localized in the proliferating zone and the hypertrophic zone (Arrow heads). The scale bar indicates 50 μm. (C) Expression levels of *Aire, Col2a1 and Col10a1* of micromass-cultured C3H10T1/2 cells treated with shAire or sh*LacZ* (negative control) and treated with BMP2 for 0, 5 and 9 days, as determined by real-time RT-PCR and normalized to *Gapdh*. Data are presented as mean ± S.D. * indicates $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

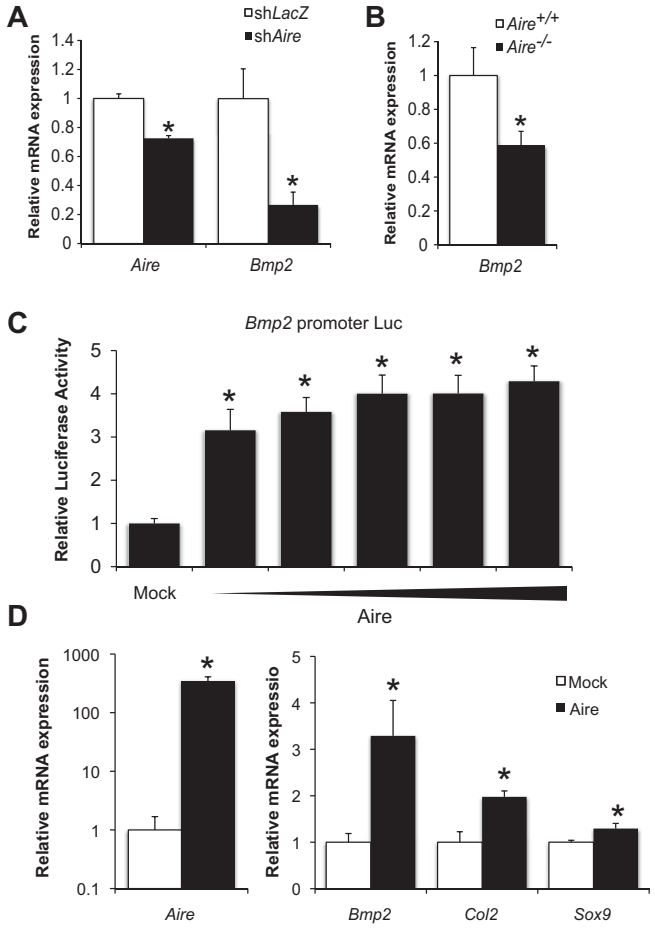

Fig. 3. Aire can facilitate chondrocyte differentiation by up-regulating *Bmp2* expression. (A) The expression of *Bmp2* was down-regulated by sh*Aire* in ATDC5 cells, as determined by real-time RT-PCR. (B) The expression levels of *Bmp2* in primary cultured chondrocytes obtained from *Aire*$^{-/-}$ mice was decreased compared to that of *Aire*$^{+/+}$ littermates, as determined by real-time RT-PCR. Data are presented as mean ± S.D. * indicates $p < 0.05$. (C) A luciferase assay was performed in ATDC5 cells transfected with the luciferase reporter vector (pGL3) including 2 kb of the *Bmp2* promoter and Aire expression vector (pcDNA3.1-Flag-Aire vector). Data are presented as mean ± S.D. * indicates $p < 0.05$ when compared to a mock transfection. (D) Expression levels of *Aire, Col2a1* and *Sox9* in micromass-cultured C3H10T1/2 cells without BMP2 treatment, as determined by real-time RT-PCR and normalized to *Gapdh*. Data are presented as mean ± S.D. * indicates $p < 0.05$.

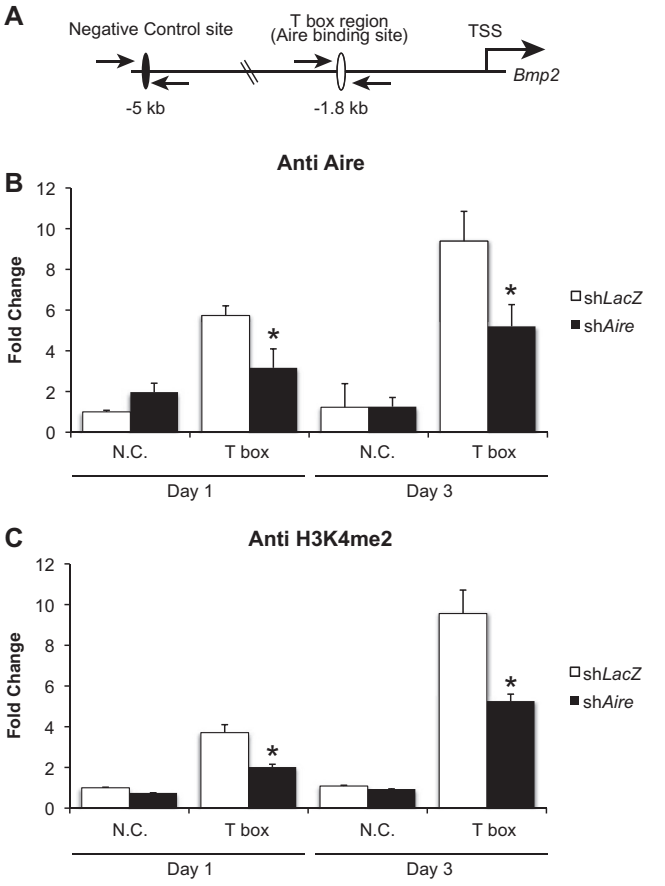

Fig. 4. Aire bound to the *Bmp2* promoter regulates *Bmp2* expression through histone H3K4 modification. (A) Schematic models of a ChIP assay for the T box and negative control sites (N.C.) in the *Bmp2* promoter region. A ChIP assay was performed using anti-Aire antibody (B) and anti-H3K4me2 antibody (C) for DNA extracted from micromass-cultured C3H10T1/2 cells treated with sh*Aire* or sh*LacZ* (negative control) and BMP2 for 1 and 3 days. Data were normalized by % input and are represented as mean ± S.D. * indicates $p < 0.05$.

the di-methylation of H3K4 since Aire contains a PHD zinc finger, which can recognize hypomethylated H3K4 [24]. As a result of a ChIP-qPCR assay, H3K4me2 was detected in the T box in the*Bmp2* promoter, and the modification levels were increased during chondrocyte differentiation. When Aire expression was knocked down, H3K4me2 levels at the T box site were decreased (Fig. 4C). Taken together, Aire may directly bind to the consensus Aire binding motif in the *Bmp2* promoter to regulate its expression through alteration of histone H3K4 methylation.

4. Discussion

In this study, we used gene expression microarrays to identify novel transcriptional and/or epigenetic factors in chondrocyte differentiation, which may give insight into the mechanism of chondrocyte differentiation. The microarray data showed that several differentially expressed genes exist during chondrocyte differentiation. In proliferative chondrocyte differentiation, the expression of Aire was significantly up-regulated. Analyses of *Aire* stable knockdown cells and *Aire*$^{-/-}$ primary cultured cells revealed that Aire may facilitate early stages of chondrocyte differentiation.

BMP2 a member of the transforming growth factor-βsuperfamily, plays a key role in inducing differentiation of MSCs into chondrocytes to form cartilage tissue via the BMP/Smad signaling pathway [25,26]. BMP2 is regulated by Gli2 in the sonic hedgehog signaling pathway. In this pathway, PI3-kinase/insulin-like growth factor can induce the expression of Gli2 [27]. Moreover, it has been documented that Aire is an active insulin regulator in thymus cells and *Bmp2* was repressed in *Aire*$^{-/-}$ thymic epithelial cells [28]. Based on this knowledge, we characterized the function of Aire on *Bmp2* expression in chondrocytes and found that Aire may play a role in the regulation of *Bmp2* expression through histone modification. These results were supported by previous reports, which suggested that the PHD1 domain of Aire can bind to hypomethylated H3K4 to concentrate Aire in binding regions and then activate the expression of target genes in mammary epithelial cells (MECs) [29].

Aire consists of a PHD zinc finger, but no significant domains harboring methyltransferase activities. However, a ChIP assay revealed that knockdown of Aire could decrease H3K4me2 levels at the Tbox site in the *Bmp2* promoter region. This alteration can be caused by histone modifiers, which interact with Aire on chromatin. It has been demonstrated that the PHD zinc finger of Aire can recognize hypomethylated histone H3 N-terminal tails to recruit transcriptional co-regulators including histone modifiers [24]. Identification of components of the Aire complex by biochemical purification may lead to comprehension of the precise molecular

mechanisms in the regulation of *Bmp2* expression by Aire during early chondrocyte differentiation.

Aire$^{-/-}$ mice did not exhibit significant abnormal phenotypes in skeletal morphology [28]. However, patients with mutations at the*AIRE* gene locus suffer from autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) and some of them show reversible metaphyseal dysplasia (RMD) with notable progressive growth and abnormal endochondral ossification in adolescents [30]. *Cst10*$^{-/-}$ mice developed and grew normally but showed abnormal phenotypes in formation of osteoarthritic osteophytes, age-related ectopic ossification and healing of bone fractures [31]. Our current study and previous reports suggest that Aire might play an important role in chondrocyte differentiation during pathological conditions such as fracture healing or osteoarthritis development. Induction of skeletal diseases into *Aire*$^{-/-}$ mice will help to further understand the precise roles of Aire in chondrocyte differentiation in pathological conditions.

Taken together, we have identified a novel regulatory factor in chondrocyte differentiation, Aire, which can modify *Bmp2* expression though alteration of the epigenetic status of the *Bmp2* promoter region. This investigation may open the window for the development of therapeutic strategies against diseases related to chondrocyte differentiation, such as osteoarthritis or fracture healing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.001.

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