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Retinoic Acid and the Transcription Factor MafB Act Together and Differentially to Regulate Aggrecan and Matrix Metalloproteinase Gene Expression in Neonatal Chondrocytes

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ABSTRACT

Vitamin A (VA) and its active form, retinoic acid (RA), are regulators of skeletal development and chondrogenesis. MafB, a transcription factor, has been identified as an important mediator in monocyte and osteoclast differentiation. However, the presence and function of MafB in chondrocytes is not clear. In this study, MafB gene expression was regulated by both the VA status of the mother (VA-marginal, adequate, and supplemented diets) and by direct oral supplementation of the neonates with VARA (VA mixed with 10% RA). Expression was highest in neonates of VA-supplemented versus VA-marginal dams ($P < 0.05$), and in VARA-treated versus placebo-treated neonates across all diet groups ($P < 0.05$). To examine cellular changes, primary chondrocytes derived from neonatal rat ribs were cultured in the presence of RA for up to 48 h. MafB mRNA exhibited a time- and dose-dependent increase in response to RA, while the induction of MafB mRNA was attenuated by BMS-493, a pan-RAR inverse agonist, implicating RAR signaling in the regulation of MafB. The genetic knockdown of MafB in chondrocytes using siRNA ($MafB^{SI}$ chondrocytes) abrogated the RA-induced increase in MafB expression. $MafB^{SI}$ chondrocytes expressed higher levels of aggrecan mRNA. Additionally, the increased matrix metalloproteinase (MMP)3 and MMP13 gene expression due to RA was attenuated in $MafB^{SI}$ chondrocytes, while total extracellular matrix staining was increased. These results support a role for MafB as a regulator of chondrocyte gene expression and matrix formation via control of aggrecan, MMP3 and MMP13 expression, and indicate an important role for RA in the regulation of MafB. J. Cell. Biochem. 114: 471–479, 2013.

KEY WORDS: CHONDROCYTES; RETINOIC ACID; MafB; AGGRECAN; MATRIX METALLOPROTEINASE

Growth plate activity is attributable to skeletal growth and function [Kronenberg, 2003; Adams et al., 2007]. During this process, chondrocytes in the proliferative zone are small in size and synthesize an abundant extracellular matrix primarily consisting of type II collagen and the large proteoglycan aggrecan. As chondrocytes differentiate, cells enlarge into pre-hypertrophic and hypertrophic chondrocytes, extracellular matrix is replaced by type X collagen; MMP3 and MMP13, which catalyze degradation of collagen and aggrecan, is increased [Fosang et al., 1996; Mitchell et al., 1996]. The matrix eventually becomes mineralized, and replaced by bone and marrow.

Vitamin A (VA) and its active form, retinoic acid (RA), are regulators of skeletal development and chondrogenesis [Zile, 2001; Clagett-Dame and DeLuca, 2002; Weston et al., 2003]. RA is produced from VA via a number of oxidative enzymes, which differ in relative expression among tissues and cell types [Ross, 2003; Napoli, 2012]. RA can induce chondrocyte terminal differentiation and alter extracellular matrix homeostasis though regulating the expression of genes involved in extracellular matrix, such as type X

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Materials disclosure: Information about the materials and methods used is available from Dr. Ross. No permanent new materials or models were generated.

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collagen, MMP3, MMP13, and aggrecan [Oettinger and Pacifici, 1990; Iwamoto et al., 1993; Jimenez et al., 2001; Yasuhara et al., 2010]. In general, RA regulates gene expression through its nuclear receptors (RARs), consisting of three subtypes, RARα, RARβ, and RARγ [Bastien and Rochette-Egly, 2004]. RARγ is the most strongly expressed of the RARs in the growth plate [Koyama et al., 1999; Williams et al., 2009]. RARγ functions in the both unliganded- and liganded forms along with the chondrocyte proliferation and differentiation [Williams et al., 2009; Yasuhara et al., 2010].

MafB is a transcription factor, belonging to the Maf (musculoaponeurotic fibrosarcoma) family [Motohashi et al., 1997]. Similar to other so-called large Maf proteins, MafB contains a basic leucine zipper structure at the carboxy-terminal portion, which mediates DNA binding and subunit dimerization, involved in the regulation of gene expression. MafB is best known as a regulator of various developmental processes, such as segmentation in the hindbrain [Grapin-Botton et al., 1998; Hernandez et al., 2004; Maves and Kimmel, 2005], and islet beta-cell differentiation [Matsuoka et al., 2003; Kataoka et al., 2004; Artner et al., 2007ab]. MafB is also an important regulator of monocytic cell differentiation function [Eichmann et al., 1997; Kelly et al., 2000; Bakri et al., 2005; Gemelli et al., 2006]. MafB has also been identified in the chondrocytes [Sakai et al., 1997]; however, the function of MafB during chondrocyte differentiation has not been studied. In the present study, we hypothesized a role for VA in vivo, and directly for its metabolite RA, in the regulation of MafB expression, and of MafB in the regulation of matrix-related gene expression. Our results show that MafB is localized in both proliferative and hypertrophic chondrocytes in the growth plate. MafB expression is increased in response to VA and/or RA treatment both in vivo and in vitro. Moreover, RA-induced MafB acts as a regulator of chondrocyte gene expression and matrix formation. Thus, MafB may be a novel regulator of interest regarding chondrocyte matrix formation and bone repair.

MATERIALS AND METHODS

ANIMALS

Animal protocols were approved by the Institutional Animal Use and Care Committee of the Pennsylvania State University. Female Sprague–Dawley rats with 1-week-old female pups were purchased from Charles River (Wilmington, MA). Briefly, upon arrival the dams were fed a controlled VA-deficient diet. To achieve differences in maternal VA status in their offspring, the weanling female rats were fed one of three levels of VA in a standard AIN-93G purified diet for 10 weeks; they were then mated and fed the same diet mating and through pregnancy and lactation. These diets are denoted VA marginal (0.4 μg retinol/g diet, VAM), VA adequate (7.67 μg retinol/g diet, VAA), and VA supplemented (104 μg retinol/g diet, VAS), n = 3–4 L per diet. The offspring of these mothers were studied at birth (P0) and on postnatal Day 7 (P7), n = 3–6 pups per litter. Additionally, half of the newborns were orally supplemented with VARA, or oil as placebo, on P1, P4, and P7. Tissues were collected 6 h after the last oral dose. VA (all-*trans*-retinyl palmitate) and RA were purchased from Sigma–Aldrich (St. Louis, MO). VA and RA were admixed (10:1 molar ratio) [Ross et al., 2006], dissolved in oil, and given orally, using a small micropipette, in a concentration that was equivalent to 11 μg of retinol and 0.6 μg of RA/g pup.

IN SITU HYBRIDIZATION (ISH)

Femurs were imbedded in optimal cutting temperature (OCT; VWR LabShop, Batavia, IL) and frozen on dry ice, then stored at −80°C. Sections were cut at 10 μm thickness in a −20°C chamber of a Shandon Cryostat, and transferred onto Probe-On Plus microscope slides (Fisher Scientific, Pittsburgh, PA), and then sections were dried briefly on a 37°C plate and stored at −80°C. ISH was carried out with digoxigenin (DIG)-labeled probes. MafB, type II collagen (Co12al), and type X collagen (Col10al) probes were prepared by using DIG RNA labeling kit (Roche Diagnostics, Indianapolis, IN). Briefly, MafB, Col2a1, and Col10a1 were amplified by standard PCR and cloned into a T easy-vector (Promega, Madison, WI). The primers used were: MafB: 5′-CAGAGCTTCGACGGCTTC-3′ (forward), 5′-GGTCCTCCACACTACCGTTG-3′ (reverse); 5′-CGGACTCCCTATCCTGTGTC-3′ (forward), 5′-TCAGCTTGCT GCTACCTTCTC-3′ (reverse); Col2a1: 5′-ACCTGGTACCCCTGGAAATC-3′ (forward), 5′-GACCATCTTGACCTGGGAAA-3′ (reverse); Col10a1: 5′-ATCCATGTGAAGGGGACTCA-3′ (forward), 5′-CCAGTGGAATAGAAGGCACA-3′ (reverse). The plasmids were linearized with a 5′-overhanging end by restriction enzymes. DIG-labeled RNAs (anti-sense and sense RNAs) were synthesized by DIG RNA Labeling Kit using the linearized DNA as templates, then hydrolyzed into 100–150 bp lengths for use.

For detection, slides were incubated in cold acetone for 10 min, then air-dried. The slices were fixed in 4% paraformaldehyde for 15 min at 4°C, rinsed with PBS twice, incubated in 0.5% (v/v) acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) for 10 min, washed with 2× SSC (20× SSC: 3 M NaCl, 0.3 M Na_3Citrate) twice, incubated in 50% formamide in 1× SSC at 60°C for 20 min, then passed through cold alcohol gradient solution, and air dried. For hybridization, slides were incubated with pre-heated (80°C for 2 min) probes in the buffer (1 mg/ml tRNA, 50% formamide, 0.3 M NaCl, 10 mM Tris–HCl, pH 6.8, 10 mM $NaPO_4$, 5 mM EDTA, 1× Denhardt's, 10% dextran sulfate) at 42°C overnight. Unhybridized probes were washed with 0.2× SSC twice at 55°C for 1 h, NTE buffer (500 nM NaCl, 10 mM Tris–Cl, pH 7.5, 1 mM EDTA) for 30 min, then incubated in NTE containing 20 μg/ml RNase at 37°C for 30 min, rinsed with NTE twice, and finally washed with 0.2× SSC at 55°C for 1 h. For signal detection, the DIG Wash and Block buffer set (Roche Diagnostics) was used. Slides were incubated in washing buffer for 5 min and then blocking buffer for 30 min, followed by incubation with alkaline phosphatase-conjugated anti-DIG antibody (1:200 dilution, Roche Diagnostics) overnight. Then the slides were washed in detection buffer for 10 min, then exposed to the substrate (BCIP/NBT, Roche Diagnostics) with 240 μg/ml levamisole in the dark for 2–4 days. The color reaction was stopped by incubation in TE buffer.

QUANTITATIVE-PCR (Q-PCR)

Femur and tibia were broken and the bone marrow was flushed out. Total RNA was extracted from bone using Trizol (Invitrogen, Carlsbad, CA) and quantified by spectrophotometry. Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA).

mRNA transcript levels were determined by real-time q-PCR, using 18S rRNA or GADPH as internal control [Zhang et al., 2010]. Primers were, for MafB: 5′-CAGAGCTTCGACGGCTTC-3′ (forward), 5′-GGTCCTCCACACTACCGTTG-3′ (reverse); aggrecan: 5′-CAACCT-CCTGGGTGTAAGGA-3′ (forward), 5′-GCTTTGCAGTGAGGATCAC-A-3′ (reverse); MMP13: 5′-CCCTCGAACACTCAAATGGT-3′ (forward), 5′-GAGCTGCTTGTCCAGGTTTC-3′ (reverse); MMP3: 5′-ACCCCACT-CACATTCTCCAG-3′ (forward), 5′-CTGACTGCATCGAAGGACAA-3′ (reverse); GAPDH: 5′-ATGGGAAGCTGGTCATCAAC-3′ (forward), 5′-GGATGCAGGGATGATGTTCT-3′ (reverse).

PRIMARY CHONDROCYTE CULTURE

Chondrocytes were prepared from 1- to 7-day old rats reared by mothers fed a stock chow diet [Lefebvre et al., 1994]. Briefly, the rib cage was dissected and rinsed in sterile PBS, incubated at 37°C for 30 min in 2 mg/ml proteinase K (Sigma–Aldrich) in PBS, then incubated at 37°C for 30 min in 3 mg/ml collagenase type II (Worthington, Lakewood, NJ) in DMEM (Invitrogen, Madison, WI). The cartilage was washed with PBS several times by repeated gentle pipetting, and separated from soft tissues by sedimentation. The cartilage was cut into small pieces and then further digested with collagenase for 3–4 h. After digestion, the cell suspension was filtered though a 45-μm cell sieve, rinsed in DMEM, then cultured in DMEM with 10% fetal bovine serum (FBS), and 100 units/ml of penicillin and streptomycin, at 37°C in a humidified 5% CO_2-air incubator.

SMALL INTERFERING RNA (siRNA) SILENCING

siRNA targeting MafB (Thermo Scientific, Lafayette, CO) was transfected into cultured primary neonatal rat chondrocytes to knock down MafB expression, designated $MafB^{SI}$ chondrocytes, using DharmaFECT tranfection reagents (Thermo Scientific) according to the manufacturer's protocol. In a preliminary study, DharmaFECT transfection reagent 1 (one of four supplied in the kit) gave the best knockdown results and was selected for use. siRNA targeting GADPH (Thermo Scientific) was used as a positive control. As a negative control we used an siRNA (Thermo Scientific), which is predicted not to target any known vertebrate gene. The cells were plated in 96-well plates at 10,000 cells per well on Day 1. After transfection (Day 2), the medium was changed to complete culture medium with antibiotics and the cells were treated with 20 nM RA on Day 3, then harvested on Day 4 or 5.

To evaluate chondrocyte differentiation, Alcian blue staining was conducted. Cultured cells were fixed with 4% (w/v) paraformaldehyde for 10 min, then incubated in 70% ethanol for 10 min, 5% acetic acid for 5 min, and stained with 1% Alcian blue (pH 1.0) for 2 h. Excess stain was washed out. Then, the stain was quantified by solubilizing the stain with 6 M guanidine hydrochloride overnight at room temperature. Absorbance at 620 nm was measured using a spectrophotometer.

STATISTICS

Results are shown as the mean ± SEM of at least n = 3 observations. Prism 5 software (GraphPad Software, La Jolla, CA) was used for analysis. Depending on design, one-way or two-way ANOVA was conducted, followed by Bonferroni's post hoc multiple comparison test to determine the statistical significance between multiple groups. A P-value <0.05 was considered statistically significant.

RESULTS

MafB IS LOCATED FROM PROLIFERATIVE ZONE TO HYPERTROPHIC ZONE IN THE GROWTH PLATE

First, to localize MafB expression in the growth plate, its mRNA was detected by ISH. The region examined is shown in a von Kossa-stained section in Figure 1A. While type II collagen was predominant in proliferative zones (Fig. 1B), and type X collagen was predominant in the hypertrophic zone (Fig. 1C), MafB mRNA was detected in both proliferative and hypertrophic chondrocytes

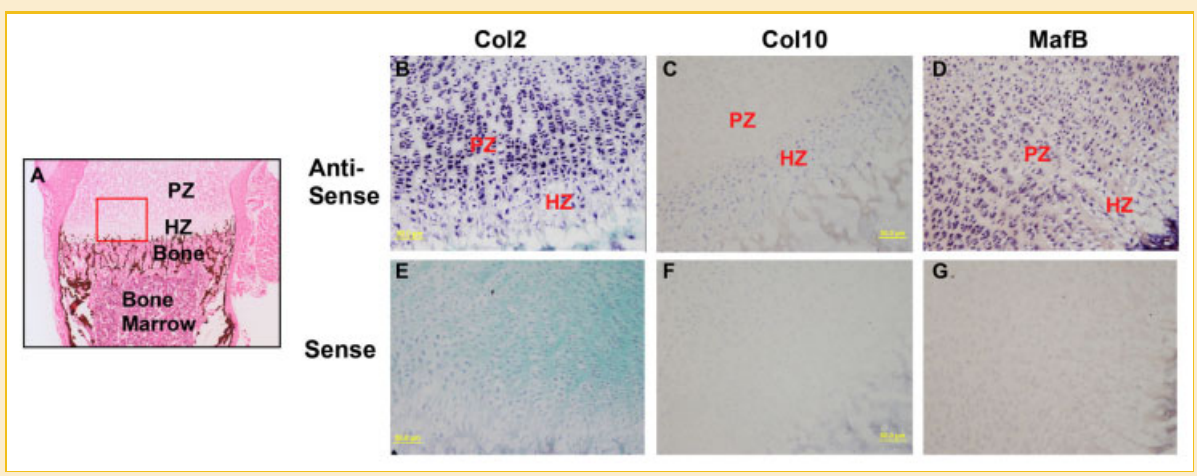

Fig. 1. MafB localization in the growth plate. A: Illustration of growth plate with von Kossa staining. A section of femur was stained with 1% silver nitrate and counterstained with nuclear fast red. Insert (red square) represents relative location of images from B to F. All sections are from femurs of 7-day old rat pups of dams fed normal diet. Type II collagen (B), type X collagen (C), and MafB (D) mRNA were detected by in situ hybridization (ISH) with antisense probes; in B and E, the slides were counterstained with methyl green. E–G: Sense probes were used as negative controls for ISH. Magnification: A, 40×; B through G, 100×. PZ, proliferative zone; HZ, hypertrophic zone.

(Fig. 1D). For each probe, the sense control showed a lack of specific staining (Fig. 1E–G).

MafB EXPRESSION IS REGULATED BY BOTH MATERNAL VA STATUS AND DIRECT ORAL VARA SUPPLEMENTATION

Maternal VA status is important for neonatal bone development. We next asked whether MafB expression in bone was affected by maternal VA status or by direct oral supplementation with VARA, a combination of VA and RA [Ross et al., 2006]. In a recent study [Zhang and Ross, 2012], we have shown that the varying the VA content of the mother's diet during pregnancy results in different levels of tissue VA in their P0 neonates: the livers of pups from VA-marginal, VA-adequate, and VA-supplemented dams equaled 20 ± 2.1, 32 ± 4.2, and 121 ± 17 nmol/g tissue, respectively, all of which differed ($P < 0.05$) from each other. Plasma retinol also differed significantly between P0 neonates from the VA-supplemented mothers (1.26 ± 0.07 μM) and pups from either the VA-marginal or VA-adequate dams (0.81 ± 0.04 and 0.94 ± 0.02 μM). Thus, exposure to VA in utero via differences in maternal dietary VA intake significantly altered the VA storage in the newborns, but only increased plasma retinol when the mother's diet was VA supplemented. For all pups that were supplemented directly with VARA, both liver and plasma retinol were increased significantly, but to the same extent for each maternal diet. Liver total retinol increased by approximately 400 nmol/g and plasma

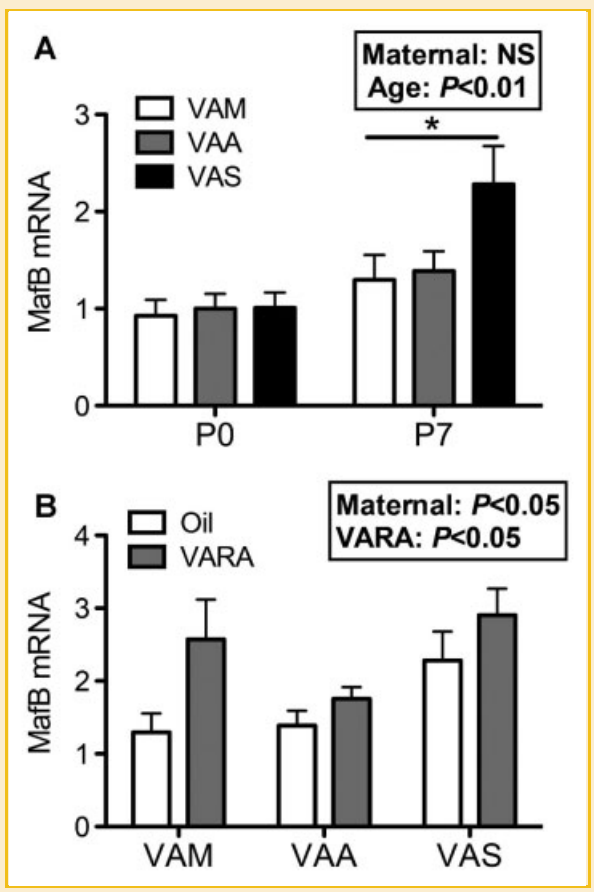

Fig. 2. MafB expression in newborn and neonatal bone. A: Bones of pups from dams with three different levels of VA status (VAM, VAA, VAS) were analyzed at P0 and P7. B: Pups from dams with different VA status were orally supplemented with oil or VARA on P1, P4, and P7, and then bones were collected 6 h after last oral dose, as described in Materials and Methods Section. MafB mRNA in bone was determined by q-PCR, with GADPH as internal control; all numbers were normalized to the P0 VAA group (defined as 1.0). Two-way ANOVA (maternal VA intake and postnatal age, or maternal VA intake and oral VARA supplementation), was conducted (shown in box), followed by Bonferroni's post hoc multiple comparison test, n = 6/group; $^{*}P < 0.05$.

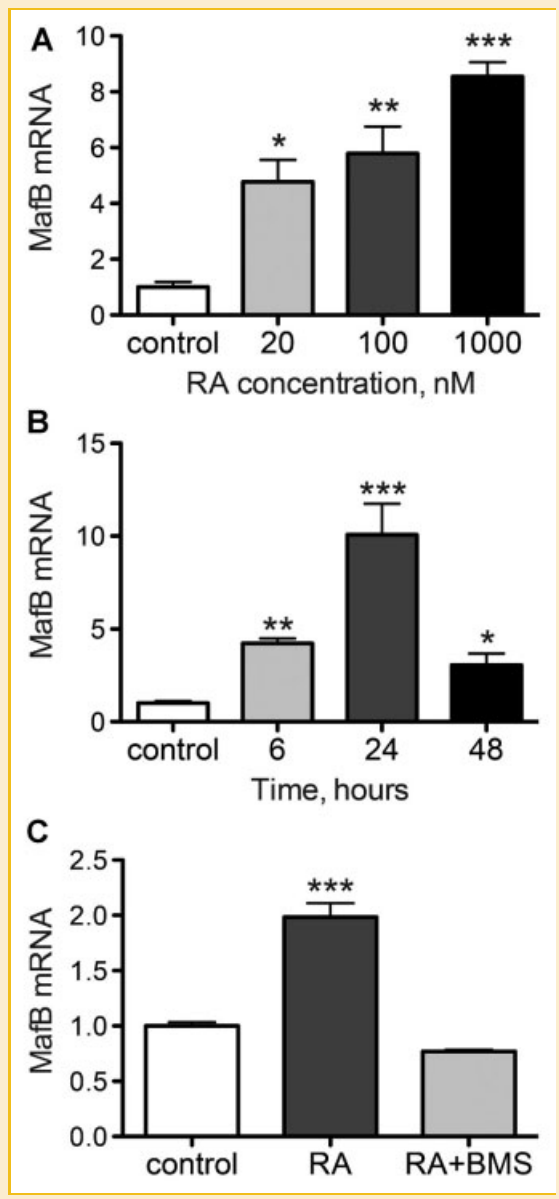

Fig. 3. MafB mRNA is upregulated by RA time- and dose-dependently in primary chondrocytes, in a RAR-dependent manner. Q-PCR analysis of MafB mRNA was conducted in primary chondrocytes. A: Cells were treated with different concentrations of RA for 24 h. B: Cells were treated with 1 μM RA for 6, 24, or 48 h. C: Cells were treated with 20 nM RA for 2 days in the absence or presence of pan-RAR inverse agonist, BMS493 (BMS, 1 μM). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle control. n = 3.

retinol by 2 μM as a result of direct treatment with VARA [Zhang and Ross, 2012].

MafB mRNA in newborn (P0) and neonatal (P7) rat bone was examined by q-PCR. It is noteworthy that MafB mRNA did not differ in P0 bone, in spite of differences in VA in liver as noted above. However, the level of MafB mRNA was significantly increased in the P7 neonatal bone of VA-supplemented mothers compared to VA-marginal mothers (Fig. 2A), suggesting that maternal diet during lactation has a greater effect on neonatal MafB expression than does the maternal diet during gestation. Treatment of the P7 neonates with VARA also resulted in an overall increase in MafB mRNA (Fig. 2B, $P < 0.05$). However, there were no differences with VARA within the three diet groups, possibly due to the small sample size per group. There also was no interaction between maternal diet and VARA, suggesting that the effect of VARA was similar in all VARA-treated groups regardless of maternal diet. Together, these results suggest that MafB is responsive to VA-dependent regulation in neonatal chondrocyte-lineage cells by direct VARA treatment in the neonatal period, or by increasing the VA intake of the lactating dam in the neonatal period. VA supplementation of the mother is known to result in higher VA levels in the mother's milk, and as a result in an increased concentration of VA in the plasma and liver of the nursing neonates [Davila et al., 1985].

RESPONSIVENESS OF MafB TO RA IS RAR-DEPENDENT

To further explore mechanisms of RA induction, cell culture experiments were performed using primary rat chondrocytes isolated from neonatal rat ribs. These chondrocytes were then treated with different doses of all-*trans*-RA for 24 h. MafB mRNA was significantly increased by RA dose-dependently, beginning as low as 20 nM RA (Fig. 3A), a concentration considered physiological in plasma. In a kinetic study, MafB mRNA was significantly increased by RA by 6 h, reaching a peak at 24 h (Fig. 3B). It is known that RA generally regulates gene expression through its nuclear receptors [Bastien and Rochette-Egly, 2004]. When chondrocytes were pre-treated with 1 μM of a pan-RAR inverse agonist, BMS493, the induction of MafB by RA was completely blocked (Fig. 3C), suggesting that the increase in MafB expression in RA-treated chondrocytes is RAR-dependent.

MafB IS A REGULATOR OF EXTRACELLULAR MATRIX HOMEOSTASIS THROUGH THE CONTROL OF AGGRECAN AND Mmps

To determine the function of MafB in RA-induced chondrocyte differentiation, we established chondrocytes with reduced MafB expression using siRNA that specifically targets MafB mRNA, which we designated MafB^{SI} chondrocytes. As a negative control, siRNA without targets was used. The MafB knockdown was verified by testing the level of mRNA. MafB mRNA was decreased by its siRNA (Fig. 4A, control cells). As in our previous experiments, treatment of control cells with RA increased MafB expression and this was completely abrogated by siRNA, as expected. Thus, the treatment was successful in modulating MafB expression. As RA is known to regulate the expression of genes involved in extracellular matrix,

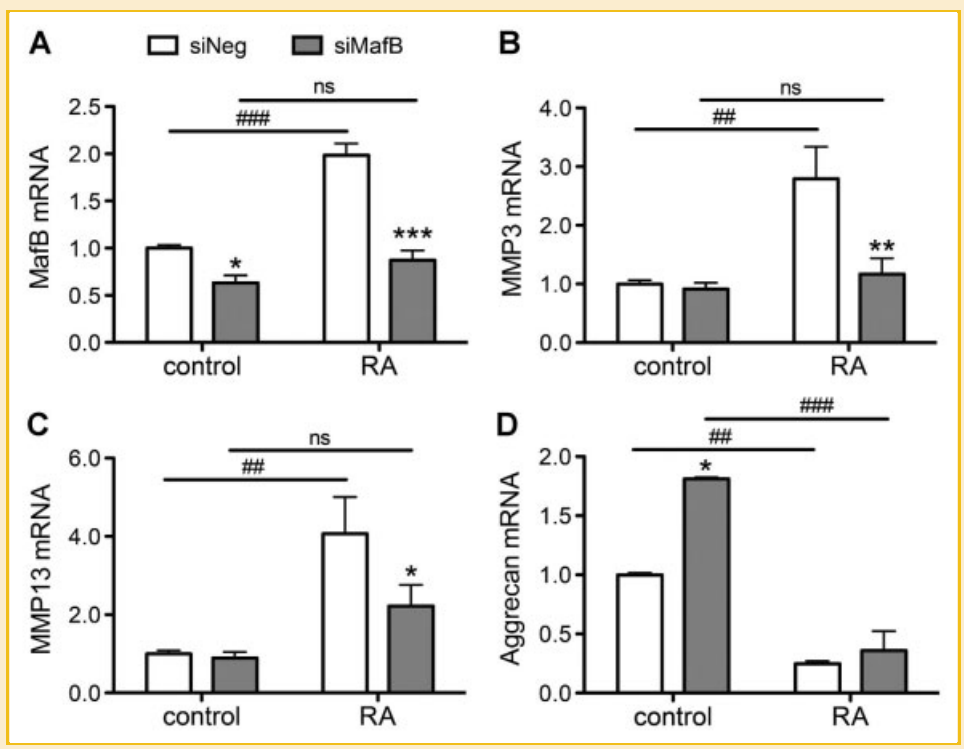

Fig. 4. Gene expression in MafB knockdown chondrocytes. Q-PCR analysis of MafB (A), MMP3 (B), MMP13 (C), and aggrecan (D) in MafB^{SI} primary chondrocytes treated with 20 nM RA for 2 days. siRNA without targets was used as a negative control. Comparison between genotypes: $^{*}P < 0.05$, $^{**}P < 0.01$. $^{\#}$Comparison between treatments: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. n = 3–6/group.

such as type X collagen, MMP3, MMP13, and aggrecan [Oettinger and Pacifici, 1990; Iwamoto et al., 1993; Jimenez et al., 2001; Yasuhara et al., 2010], these genes were examined in MafB^{SI} chondrocytes and negative control cells. Neither type II nor type X collagen expression was altered by MafB knockdown (data not shown). However, MafB^{SI} chondrocytes exhibited attenuated RA-induced expression of MMP3 (Fig. 4B) and MMP13 (Fig. 4C). For these genes, there was no difference in the control-treated cells, which may suggest that MMP3 and MMP13 mRNAs are relatively stable in these cells. Moreover, in untreated chondrocytes, the reduced MafB expression in the MafB^{SI} knockdown cells resulted in increased aggrecan expression (Fig. 4D), while RA reduced aggrecan expression by more than 75% regardless of silencing of MafB (Fig. 4D).

PROTEOGLYCAN ACCUMULATION IS REDUCED BY RA AND PROPORTIONAL TO AGGRECAN EXPRESSION

Aggrecan and MMP13 are important factors for extracellular proteoglycan synthesis and degradation [Fosang et al., 1996; Caterson et al., 2000]. Therefore, we determined the chondrocyte phenotype, monitoring proteoglycan accumulation by quantification of Alcian blue staining. RA induces chondrocyte differentiation and decreased extracellular matrix, thereby the staining was decreased (Fig. 5A). However, MafB^{SI} chondrocytes exhibited an increase of Alcian blue staining (Fig. 5A), which was highly correlated with the level of aggrecan gene expression in the same cells (Fig. 5B, $P < 0.0001$). Therefore, these results suggested a role of MafB in the regulation of cartilage matrix formation, in which reduced aggrecan gene expression, as shown above in Fig. 4D, and reduced proteoglycan accumulation (Fig. 5A) play roles.

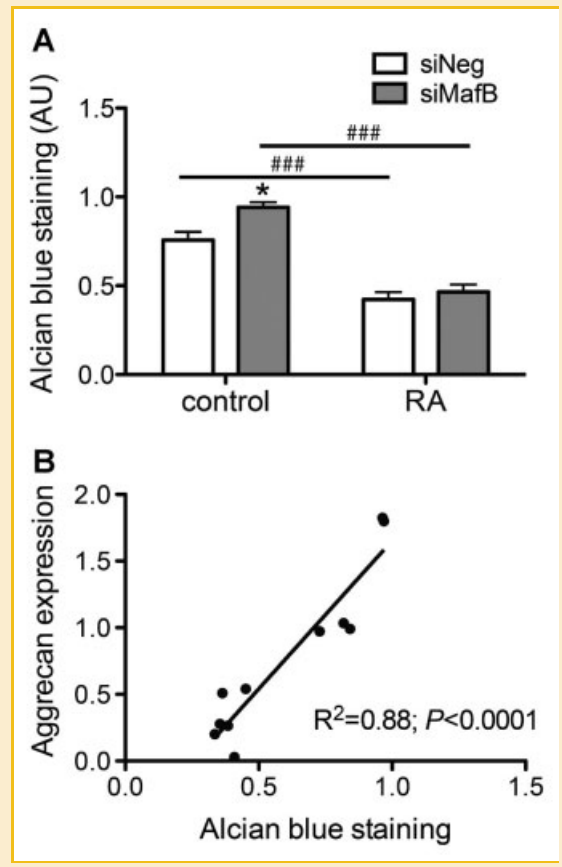

Fig. 5. Proteoglycan staining in chondrocytes with reduced MafB expression. A: MafB-silenced (MafB^{SI}) primary chondrocytes were treated with 20 nM RA for 2 days and then were stained with 1% Alcian blue, which was quantified (OD620 nm) after solubilizing the stained cells with guanidine hydrochloride. *Comparison between genotypes: $^{*}P < 0.05$. #Comparison between treatments: $^{\#\#\#}P < 0.001$. n = 5. B: Correlation analysis of Alcian blue staining versus aggrecan mRNA expression in MafB silencing experiments.

DISCUSSION

In present study, we studied the role of the transcription factor MafB in RA-induced chondrocyte differentiation. Here, we found that MafB is expressed in growth plate, and that MafB expression was responsive to VA and/or RA in vivo in the bone of neonates, and in vitro in cultured chondrocytes from neonatal rat ribs. The increase in MafB expression due to RA was apparently RAR-dependent, since the retinoid receptor inverse agonist BMS-493 completely blocked the RA-induced increase in MafB expression. Moreover, the reduction in MafB mRNA observed in MafB^{SI} chondrocytes resulted in a higher level of aggrecan expression and an attenuation of RA-induced expression of the matrix metalloproteinases MMP3 and MMP13, suggesting that MafB is a regulatory transcription factor in the process of neonatal cartilage formation. Overall, our results suggest a model (Fig. 6), in which RA-induced MafB functions as a regulator of chondrocyte gene expression and cartilage matrix homeostasis. The results raise a caution that the exposure to too much preformed VA (retinol), or direct exposure of the neonate to RA, can potentially "push" developing chondrocytes towards an unhealthy phenotype with an excess of MMP expression and too little aggrecan expression.

MafB is best known as a regulator of early embryonic development and cell differentiation [Cordes and Barsh, 1994; Grapin-Botton et al., 1998; Kelly et al., 2000]. The involvement of MafB in bone has not been explored. However, it is known that *kreisler* mice, in which a kr/MafB mutation leads to a functional deletion of MafB, experience abnormal sensory organ, and inner ear development [Choo et al., 2006], suggesting global otic patterning is perturbed by abnormal hindbrain patterning and kr/mafB mutation. However, whether chondrocyte differentiation is involved has not been established. In the present study, MafB expression in neonatal rat bone was affected both by the VA in the maternal diet, which was evident in the offspring at P7 but not P0, and by oral direct supplementation to the neonates with VARA. This suggests that the vulnerable period regarding maternal diet is the period of lactation when VA is transferred in milk to the nursling pups. In recent studies, we have shown that milk total retinol is increased ~sixfold in the dams fed VA-supplemented diet, while plasma retinol is also significantly elevated in the pups of VA-supplemented dams, and nearly doubled after treatment with VARA, regardless of the level of VA in the mother's diet [Zhang and Ross, 2012]. MafB expression also was increased in vivo in a similar dose- and time-dependent manner by RA. All-*trans*-RA is well characterized as a ligand for

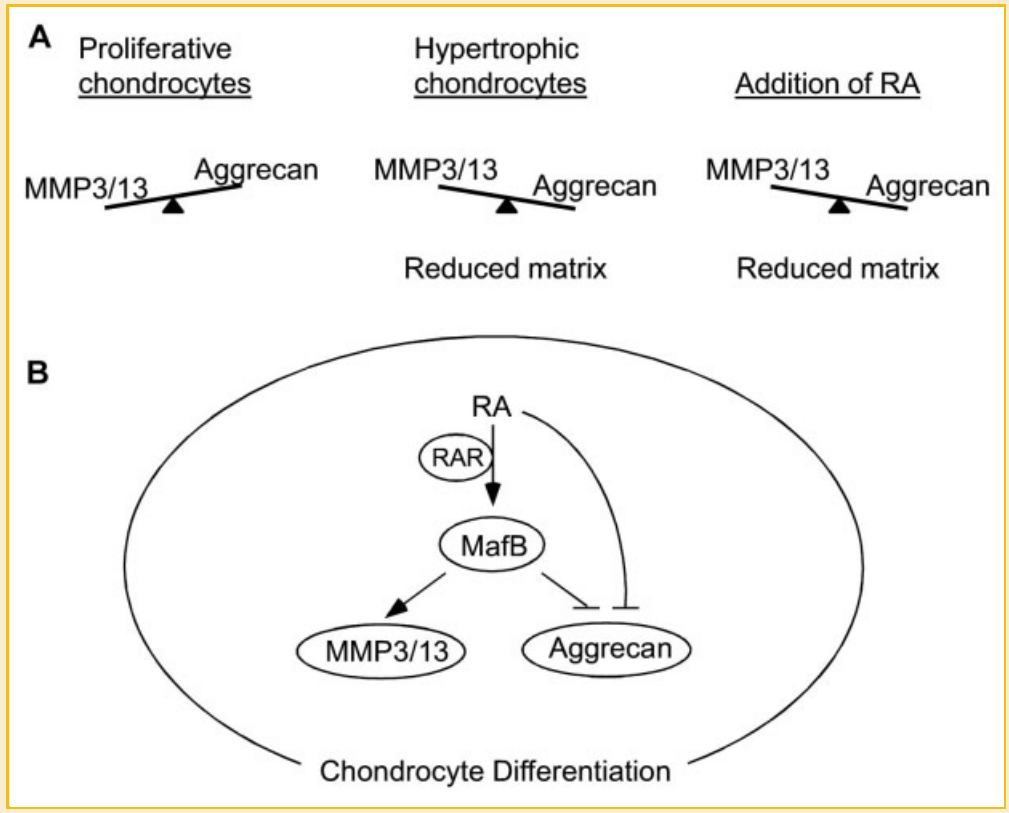

Fig. 6. Model of role of RA and MafB in the formation of cartilage matrix during chondrocyte differentiation. A: With chondrocyte differentiation, the balance of extracellular matrix is changed. RA can induce chondrocyte terminal differentiation and alter extracellular matrix homeostasis. Exogenous addition of RA can reduce aggrecan, and increase MMPs, such as MMP3, MMP13, resulting in a decrease of extracellular matrix content. B: MafB expression was induced by RA in a dose- and time-dependent manner, which was apparently RAR-dependent. Moreover, the reduction in MafB expression observed in MafB^{SI} chondrocytes resulted in increased aggrecan expression and attenuated RA-induced expression of MMP3 and MMP13. Furthermore, RA reduced aggrecan expression regardless of silencing of MafB, suggesting RA inhibits aggrecan through multiple pathways. Overall, these results implicate MafB as a regulator of chondrocyte gene expression and matrix formation via the control of aggrecan, MMP3 and MMP13 expression, and indicate an important role for RA in the regulation of MafB.

nuclear receptors RARα, β, and γ, which dimerize with retinoid X receptors and bind to specific DNA sites, known as retinoic acid response elements (RARE) [Wei, 2003; Bastien and Rochette-Egly, 2004]. Although an analysis of the MafB promoter did not result in locating a classical DR2 or DR5, we did identify two hexameric motifs separated by seven nucleotides, which could be RAR/RXR binding sites, since RAR/RXR heterodimers have been shown to be able to bind to motifs for which the spacing between the hexanucleotide repeats is not limited to either two or five nucleotides [Wang et al., 2002]. A direct effect of RA is suggested by inhibition of the response to RA in actinomycin D-treated cells (data not shown), however additional experiments on the mechanism by which RA increases MafB expression are needed.

Aggrecan is a large proteoglycan that comprises a major structural component of cartilage, particularly articular cartilage [Kronenberg, 2003; Adams et al., 2007]. It is responsible for hydrating cartilage, rendering the bone capable of resisting physical loading, and thereby aggrecan plays a major role in maintaining cartilage integrity and normal functions [Hardingham and Fosang, 1992]. The depletion of aggrecan leads to a decrease of compressibility and resilience during joint loading. Over time, this process can result in irreversible cartilage damage. The increased catabolism of aggrecan is a key pathological mechanism in arthritis [Arner et al., 1999; Yasuda, 2006; Huang and Wu, 2008]. Metalloenzymes including MMP3 and MMP13 catalyze the degradation of collagen and aggrecan, and are induced in terminally differentiated chondrocytes in the growth plate [Fosang et al., 1996; Mitchell et al., 1996], where they participate in the remodeling process essential for bone growth. Our results, as well as previous studies by other groups [Jimenez et al., 2001; Ilic et al., 2007; Yasuhara et al., 2010], indicate that RA can reduce aggrecan and increase MMP3 and MMP13 in cell culture. In our current studies, knocking down of MafB resulted in an increase of aggrecan expression in untreated chondrocytes, whereas the inhibitory action of RA on aggrecan expression was not reversed by reducing MafB in MafB^{SI} cells. These results suggest that MafB is a negative regulator of aggrecan synthesis. Moreover, we showed that knocking down MafB completely attenuated RA-induced MMP13 expression. Analysis of the MMP13 promoter by the Transcription Element Search Software reveals that, although no Maf consensus response elements (MAREs) were identified, there are multiple potential AP-1 binding sites in the upstream regulatory region of the MMP13 gene. MARE sequences are related to the AP-1 site [Blank and Andrews, 1997]. An AP-1 binding site is predicted at −73 bp in

the promoter of human MMP13, which has been characterized, where c-Fos and c-Maf can bind in inflammatory conditions [Benderdour et al., 2002; Li et al., 2010]. In addition, the region containing the AP-1 site is conserved between human and rat. Thus, the regulation of MMP13 expression by MafB could potentially also be regulated though this binding site. Together with changes of MafB that we have observed in vivo, these results suggest that MafB is involved in extracellular matrix formation and RA-induced chondrocyte differentiation.

In addition, MafB can form a heterodimeric complex with c-Fos [Blank and Andrews, 1997]. C-Fos plays an important role in chondrogenesis. Overexpression of c-Fos in ATDC5 cells was shown to inhibit chondroprogenitor cell differentiation in vitro [Thomas et al., 2000], while ectopic expression of Fos in developing chicken limb buds resulted in truncation of the cartilage in the long bones due to chondrodysplasia caused by a delay in precartilagenous condensation and severely retarded terminal differentiation of these cells into hypertrophic chondrocytes [Watanabe et al., 1997]. During the differentiation of osteoclasts, MafB is considered as a negative regulator, which interferes with the DNA-binding ability of c-Fos by dimerization, as well as other factors. Moreover, RA inhibits the differentiation of oseoclasts by inducing MafB and inhibiting c-Fos [Conaway et al., 2009]. Our preliminary results showed that RA also inhibited c-Fos expression in primary chondrocytes culture (data not shown). It can be speculated that a similar regulation on MafB exists in chondrocytes. In further studies, it will be interesting to investigate whether MafB and c-Fos interact during RA-induced chondrocyte differentiation.

Currently there is great interest in how early-life nutritional exposures, including VA, affect outcomes throughout life [Ross, 2005]. Excess VA and RA are known risk factors for bone fracture, but their significance at moderate levels and for short times, such as in this study, in early life has not been clarified, nor has attention been focused on development of osteoarthritis later in life. It should be noted that our results do not suggest that safe sources of VA, such as carotenes present in fruits and vegetables [Grune et al., 2010], are likely to pose a risk to neonatal bone formation. However, due to the significant roles of aggrecan and MMPs in regulating the composition and mechanical properties of cartilage, and because dysregulation of aggrecan and MMPs may contribute to skeletal diseases such as arthritis, further studies of RA-regulated MafB expression in models of both developing and aging cartilage now appear to be warranted.

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