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Mutations and promoter SNPs in RUNX2, a transcriptional regulator of bone formation

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Abstract

Cleidocranial dysplasia (CCD) is a dominantly inherited skeletal malformation syndrome with high penetrance and variable expressivity. It is caused by loss of function mutations in the *RUNX2* gene that encodes for a transcription factor essential for osteoblast differentiation and chondrocyte maturation. To identify new pathogenic mutations associated with CCD we screened 38 CCD patients for mutations in the *RUNX2* coding sequence. We also report the mutation screening of the "bone-related" *RUNX2* promoter in CCD patients without mutation in the *RUNX2* coding region. We identify eight new and three previously described mutations in the *RUNX2* gene. Additionally, a total of five sequence variants in the *RUNX2* promoter were detected. Three of them occur within putative zinc finger transcription factor binding sites. DHPLC analysis of chromosomes from the control population and CCD patients showed that two promoter sequence variants were unique for CCD families. Electrophoretic mobility shift assay (EMSA) with protein extracts from ROS17/2.8 and C3H10T1/2 cell lines demonstrated that the promoter sequence variants altered DNA–protein binding specificity. Moreover, one of the variants significantly decreased the expression of a *RUNX2* reporter gene in osteoblastic ROS17/2.8 cells, but not in multipotent, mesenchymal C3H10T1/2 cells. Interestingly, one of these sites bound the TRPS1 transcription factor and we demonstrated that TRPS1 is able to repress the *RUNX2* promoter. The in vitro functional studies in conjunction with analysis of clinical phenotype of CCD patients suggest that these promoter sequence variants may affect transcriptional activity of the *RUNX2* gene. Analysis of the promoter variants and RUNX2-interacting proteins may help to identify important *cis*-elements and *trans*-factors that regulate the RUNX2 transcriptional network and identify new susceptibility markers for more common bone disorders.

Keywords: Cleidocranial dysplasia; RUNX2; SNP; TRPS1

Introduction

Cleidocranial dysplasia (CCD; MIM# 119600) is a dominantly inherited disorder characterized by delayed endochondral and intramembranous ossification. Skeletal abnormalities include short stature, hypoplastic clavicles, large fontanelles, and dental abnormalities [1]. CCD exhibits a wide clinical spectrum ranging from a mild form characterized by isolated dental anomalies, to classical CCD characterized by the triad of cranial, clavicular, and dental findings, to a severe phenotype with skeletal deformity including scoliosis, osteopenia, and recurrent fractures [2–5]. CCD is caused by haploinsufficiency of the RUNX2 transcription factor [6,7]. RUNX2 is one of three mammalian orthologs of the *Drosophila*

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melanogaster Runt gene [8]. It encodes a transcription factor essential for osteoblast differentiation and chondrocyte maturation [9–11]. RUNX2 contains three domains (Fig. 1A): (i) an N-terminal Q/A domain composed of a stretch of 23 consecutive glutamines followed by 17 consecutive alanines; (ii) a RUNT domain, that has the unique ability of mediating DNA binding and protein heterodimerization; (iii) a C-terminal PST (proline/serine/threonine-rich) activation domain that can also mediate protein–protein interaction [12]. The majority of described mutations are missense mutations located in the conserved RUNT domain. These mutations abolish RUNX2 binding to the target OSE2 element (osteoblast-specific *cis*-acting element) in osteoblast-specific genes [5,6]. These and additional nonsense, splicing, and frame-shift mutations are detected in over 65% of CCD patients [13–16]. Submicroscopic deletions and chromosomal rearrangements occur in a small fraction of CCD patients [17,18].

Since RUNX2 loss of function leads to CCD, we hypothesize that promoter variants of *RUNX2* may either cause CCD or modify the phenotype. For proper skeletal development and homeostasis, *Runx2* must be regulated in strict temporal and spatial fashion. In mouse models, abnormal *Runx2* expression at the time of bone formation or remodeling exhibit clear phenotypic consequences [19–22]. The *Runx2* gene contains two separate promoters designated P1 and P2 [23]. In osteoblasts, *Runx2* expression is driven by the distal "bone-related" P1 promoter [24,25]. It has been demonstrated that a number of growth factors, signaling molecules and their receptors including members of the TGF-β superfamily, FGFR (fibroblast growth factor receptor), steroid hormones and transcription factors affect Runx2 expression and/or *Runx2*-dependent osteoblast differentiation [26–41]. Recent studies demonstrated also that targeted deletion of P1 promoter and exon 1 in mice leads to failure of formation of posterior cranium and other bones formed through endochondral ossification [42]. However, the number of described *trans*-acting factors that directly interact with the *Runx2* promoter is limited [43–46].

Here, we present the results of a screen of the *RUNX2* coding sequence and bone-related promoter in CCD patients. We hypothesized that promoter mutations affecting binding of transcription factors critical for the *RUNX2* expression might change the transcriptional activity of the gene. We analyzed CCD patients for whom we did not detect mutations in the *RUNX2* coding region to test whether mutations in the *RUNX2* promoter could contribute to the CCD phenotype. We detected *RUNX2* promoter sequence variants that altered the promoter characteristics in vitro, however, they do not cause the CCD phenotype. Analysis of these variants may help identify *cis* elements that specify transcriptional control of *Runx2* expression as well as new susceptibility markers for more common phenotypes such as osteoporosis.

Materials and methods

Patients

Patients with a clinical diagnosis of cleidocranial dysplasia were recruited via consultation services from USA, Canada, and Europe. All samples were obtained following informed consent for DNA testing. DNA samples for population studies were obtained from Baylor Polymorphism Resources. Genomic DNA was extracted from peripheral blood using standard methods.

Mutation analysis

To identify unknown mutations in the *RUNX2* gene we sequenced DNA fragments amplified by PCR. The *RUNX2* exons were amplified using intron- and exon-specific primers as described previously [6].

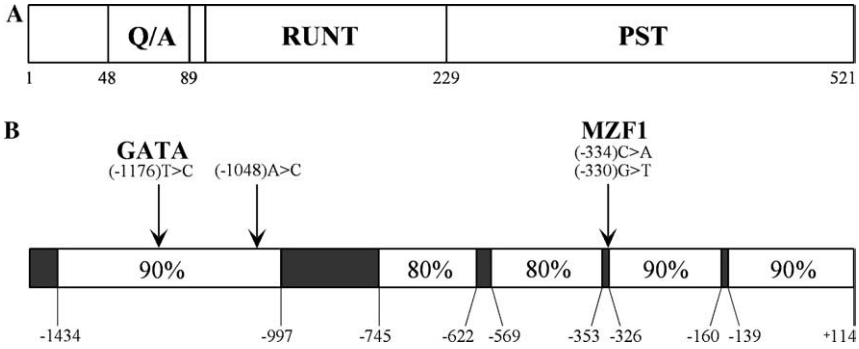

Fig. 1. (A) Domain structure of the RUNX2 protein. Q/A, polyglutamine–polyalanine domain; RUNT, DNA binding domain; and PST, proline/serine/threonine-rich domain. Numbers below the protein indicate position of amino acids at the domain junction. (B) Schematic representation of regions homologous between the human and mouse *RUNX2* promoters: open boxes, regions of high homology, percentage of homology is indicated in the boxes; black boxes, regions unique for the human promoter. Positions of unique/homologous sequence junctions are marked below the promoter. Positions of detected promoter sequence variants are indicated by arrows.

Table 1
Primers used for the *RUNX2* promoter analysis

Promoter fragment	Primer forward (5′ → 3′)	Primer reverse (5′ → 3′)	Amplicon (bp)	Analysis
Distal	CTCCATCGCTCCCAACTGATG	GCAGAGCTCTGCAGTTAAGG	1015	Mutation screening
Distal	TGAGTTCTATACATACTTGTCCA	GAATAGAGTGGGTTAACAGAAGCT	304	DHPLC
Distal	GAAGATCTGCTATCAGAAACT	—	—	Sequencing
Proximal	GCCACCCAGCAAATATGAAGCA	CTGCTTGCAGCCTTAAACTG	870	Mutation screening
Proximal	GCCACCCAGCAAATATGAAGCA	GTGACTCATGAGGATTTGGATAGA	287	DHPLC
1.3 kb	CAACGCGTCTCCATCGCTCCCAACTGATG	CCTCGAGTCACACAATCCAAAAAAGC	1316	Expression

The *RUNX2* promoter was amplified as two overlapping fragments—distal (1015 bp) and proximal (870 bp), under the following conditions: 95 °C for 10 min, followed by 35 cycles at 94 °C for 1 min; 55 °C for 1 min; 72 °C for 1 min; and a terminal extension at 72 °C for 10 min. Primer sequences for the *RUNX2* promoter are provided in Table 1.

DNA sequencing was carried out in both directions using PCR primers. An additional internal forward primer was used to sequence the distal fragment of the *RUNX2* promoter. Sequencing reactions were carried out using an ABI PRISM BigDye Terminator Cycle Sequencing kit ver. 1.0 (Applied Biosystems, Warrington, UK) and the products were analyzed on the ABI377. Each mutation was confirmed by sequencing of a PCR product from an independent PCR amplification of the affected fragment. In the case of a frame shift mutation, PCR products were sequenced following their subcloning into the TA-vector (Invitrogen, Carlsbad, CA).

The frequency of detected sequence variants in a control group was analyzed using denaturing high performance liquid chromatography (DHPLC) on a WAVE DNA Fragment Analysis System (Transgenomic, San Jose, CA). The DNA duplexes were created by heating unpurified PCR amplicons at 95 °C for 5 min and cooling slowly 1.5 °C/min to room temperature. The amplicons were run at pre-determined optimal temperatures: 57 °C for the (−1176)T > C, (−1048)A > C, (−334)C > A, and (−330)G > T substitutions. The results of chromatograms were compared for variation in shape or retention time.

Fluorescent in situ hybridization (FISH) was performed with BAC DNA (AL358135) probes on metaphases of peripheral blood lymphocytes and Epstein–Barr virus transformed lymphoblasts according to a modified procedure of Shaffer et al. [47].

DNA binding analysis

The impact of the *RUNX2* promoter sequence variants on *RUNX2* expression was assessed via transcriptional activity and DNA–protein binding properties of the detected sequence variants. Electromobility shift assay (EMSA) was carried out as previously described with double-stranded oligonucleotide probes and nuclear proteins extracts from ROS17/2.8, COS7, and C3H10T1/2 cell lines [48]. The probes were end-labeled with [α-P^{32}]dCTP using Klenow polymerase. The sequences of the oligonucleotide probes are listed in Table 2. For the super-shift nuclear protein extracts were incubated with 2 μg of anti-V5 antibody (Invitrogen, Carlsbad, CA) on ice for 15 min prior to addition of a DNA probe. Competitions were performed with 100-fold molar excess of unlabeled competitor DNA.

Table 2
DNA probes used for EMSA

Probe	Sequence (5′ → 3′ sense strand)
MZF-WT	GATCCAGGTTGAGCGGGGAGTAGAAAGA
MZF-A	GATCCAGGTTGAGAGGGGAGTAGAAAGA
MZF-T	GATCCAGGTTGAGCGGGTAGTAGAAAGA
GATA-WT	GATCCAAAAAAGGAGATAGTTTCCCAAAA
GATA-C	GATCCAAAAAAGGAGACAGTTTCCCAAAA
1048-WT	GATCCCAAAGCTTCCATTAGAAACAAAA
1048-C	GATCCCAAAGCTTCCCTTAGAAACAAAA
Non-specific competitor	GATCCCCTTGACCATAACCGTCTTCACA

Transient transfection

Expression studies were conducted in which the *RUNX2* promoter sequence variants were amplified using primers containing linkers with recognition sites for *Mlu*I (forward primer) and *Xho*I (reverse primer) endonucleases. Amplicons, after *Mlu*I and *Xho*I digestion, were subcloned into pGL3 Luciferase Reporter Vector (Promega, Madison, WI). Transient transfection experiments in ROS17/2.8 and C3H10T1/2 cell lines were performed using Lipofectamine 2000 according to the manufacture's recommendations (Invitrogen, Carlsbad, CA). The pSV-β-galactosidase vector (Promega, Madison, WI) was co-transfected as an internal control for transfection efficiency. Luciferase and β-galactosidase activities were assayed 48 h after transfection as previously described [5]. Transfections were performed in triplicate and at three doses of reporter plasmids to ensure a linear dose–response curve.

Results

Mutations and allelic variants of the RUNX2 gene

A screen for mutations in the *RUNX2* coding region (GenBank: AF001450) was performed in 38 patients from 35 families. These subjects were unselected and consecutively referred with the potential diagnosis of CCD. A total of 11 different mutations were identified in 17 CCD patients (Table 3). Two recurrent mutations (R225Q and R391X), one previously reported R190Q substitution, and eight novel mutations were detected in our group of patients. Nine mutations were de novo and two were familial. The R391X nonsense mutation was detected in a family with three affected individuals and the c.716delC frame shift mutation was detected in a family with two affected individuals. All identified *RUNX2* mutations were associated with classic CCD presentation, i.e., the triad of hypoplastic clavicle, delayed closure of fontanel, and supernumerary dentition.

To identify variants that might impact the expression level of the *RUNX2* gene, we screened the 1.3 kb fragment of the *RUNX2* bone-specific promoter together with the 5′ untranslated region containing elements regulating *RUNX2* transcriptional activity. The analysis was performed in 26 CCD patients without mutations in the *RUNX2* coding sequence. This group includes 21 patients from the current study and five patients analyzed for mutations previously. A total of five sequence variants in the *RUNX2* promoter were detected among 26 CCD patients. Virtually all analyzed samples from CCD patients demonstrated a length polymorphism of −569(T)11_13 sequence. We identified three alleles for this sequence containing 11, 12, and 13 thymidine residues (data not shown). The remaining four promoter variants represent single nucleotide polymorphisms (SNPs): (−1176)T > C, (−1048)A > C, (−334)C > A, and (−330)G > T. Alignment of the 1.5 kb human and mouse *RUNX2* promoter shows several highly homologous regions. Furthermore, the human promoter contains a region of approximately 250 bp that does not have a counterpart in the mouse promoter (Fig. 1B). Two of the detected alterations, (−1176)T > C and (−1048)A > C, are located in the region of high homology, while (−334)C >A and (−330)G > T are in a region unique for the human promoter.

To test if these SNPs might contribute to the CCD phenotype, we first compared the frequency of all SNPs in the control population and in the CCD patients (Table 4). Overall, we found one common SNP, two rare SNPs and one unique sequence variant. An

Table 3
RUNX2 mutations identified in CCD patients included in this study

Nucleotide change (cDNA)[a]	Predicted protein or RNA change	Location	No. of cases in this study	Reference
c.278insT	Frame-shift	Q/A domain	1	Present
c.386A > G	H129R	RUNT domain	1	Present
c.569G > A	R190Q	RUNT domain	1	[5]
c.604delA	Frame-shift	RUNT domain	2	Present
c.674G > A	R225Q	RUNT domain/NLS[b]	1	[5,63]
c.716delC	Frame-shift	PST domain	2	Present
c.860-2A > C	Exon skipping	PST domain	1	Present
c.878C > T	Q290X	PST domain	1	Present
c.1171C > T	R391X	PST domain	5	[5,16,64]
c.1250insA	Frame-shift	PST domain	1	Present
c.1550delTTTG	Frame-shift	PST domain	1	Present

[a] Nucleotide numbers from ATG start codon of the GenBank reference sequence AF001450.
[b] NLS-nuclear localization signal.

Table 4
Frequency of the promoter sequence variants and their in vitro effect on *RUNX2*

SNP	Transcription factor binding site	Frequency			In vitro effect	
		Population 180 chromosomes (%)	CCD without mutation 51 chromosomes (%)	CCD with mutation 64 chromosomes (%)	EMSA	Promoter activity
−1176T > C[a]	TRPS1	0	2	0	Abolished binding to TRPS1	No changes
−1048A > C	—	1	4	0	Abolished binding to LMW proteins	Decreased in ROS17/2.8
−334C > A[b]	MZF-1	0	2	1.5	Increased binding	No changes
−330G > T	MZF-1	15	20	17	Abolished binding	No changes

[a] Allele found only in one family.
[b] Allele found in two families.

(−1048)A > C transversion was detected in two unrelated CCD patients as well as in 1% of the control group. This rare SNP is not located within a consensus DNA–protein binding sequence according to the TFSEARCH database. Computer analysis with the program TFSEARCH ver.1.3 suggested that the remaining three promoter sequence variants, (−1176)T > C, (−334) C > A, and (−330)G > T, are located within binding sites of zinc-finger transcription factors. For all alterations in the *RUNX2* promoter, we performed electromobility shift assays (EMSA) to test whether the detected sequence variants identified protein binding sites and whether the variants altered the pattern of DNA binding in osteoblastic vs. nonosteoblastic cells. In addition, we studied the transcriptional activity of promoters harboring the detected single nucleotide substitutions.

Two sequence variants, (−334)C > A and (−330)G > T (Fig. 2A), occurred within a binding site for MZF1 (myeloid zinc-finger transcription factor 1). MZF-1 is a transcriptional activator in cells of hematopoietic origin, while in non-hematopoietic cells it acts as a transcriptional repressor [49]. The (−334)C > A substitution was found in one CCD patient and the (−330)G > T substitution was detected in ten CCD patients. DHPLC analysis showed that the frequency of the (−330)T allele is 15% in general population. The (−334)A allele was found in one CCD patient without another detectable *RUNX2* mutation and in one CCD family (family 102-09) in which it cosegregated with IVS5 + 1G > T mutation (Fig. 2B). Segregation analysis of the (−334)C > A substitution in the family of the patient without *RUNX2* mutation (family 102-43) revealed this alteration in the affected child, the mother and an unaffected sister of the proband (Fig. 2C). This excludes the (−334)C > A substitution as a disease causing mutation, though it could have a modifying effect on *RUNX2* expression. Interestingly, the mother carries on her other allele another sequence variant, the (−330)G > T substitution, that is located within the same MZF1 binding site as the (−334)C > A transversion. FISH analysis detected two copies of the *RUNX2* gene in the CCD patient with the (−334)C > A substitution (data not shown) thereby ruling out a deletion of one copy of the gene.

In one CCD patient a (−1176)T > C substitution, located within a GATA transcription factor consensus sequence, was identified (Fig. 3A). GATA proteins are involved in hematopoiesis, heart formation and endo-

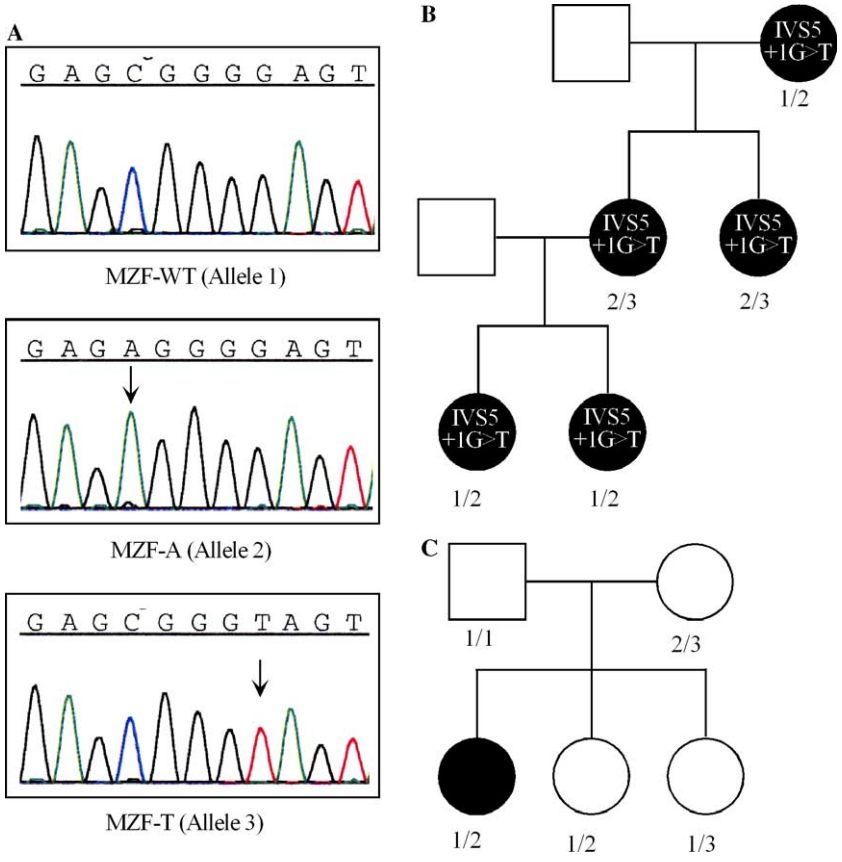

Fig. 2. Three sequence variants located within the MZF-1 consensus binding sequence in the *RUNX2* promoter. The most common variant was designated as allele 1 and the substitutions (−334)C > A and (−330)G > T as allele 2 and 3, respectively. (A) Electropherograms of the sequence variants; arrows indicate sequence changes. (B) Pedigree of the CCD family 102-09, in which the IVS5 + 1G > T mutation cosegregates with MZF-A allele. (C) Segregation of the alleles in family 102-43.

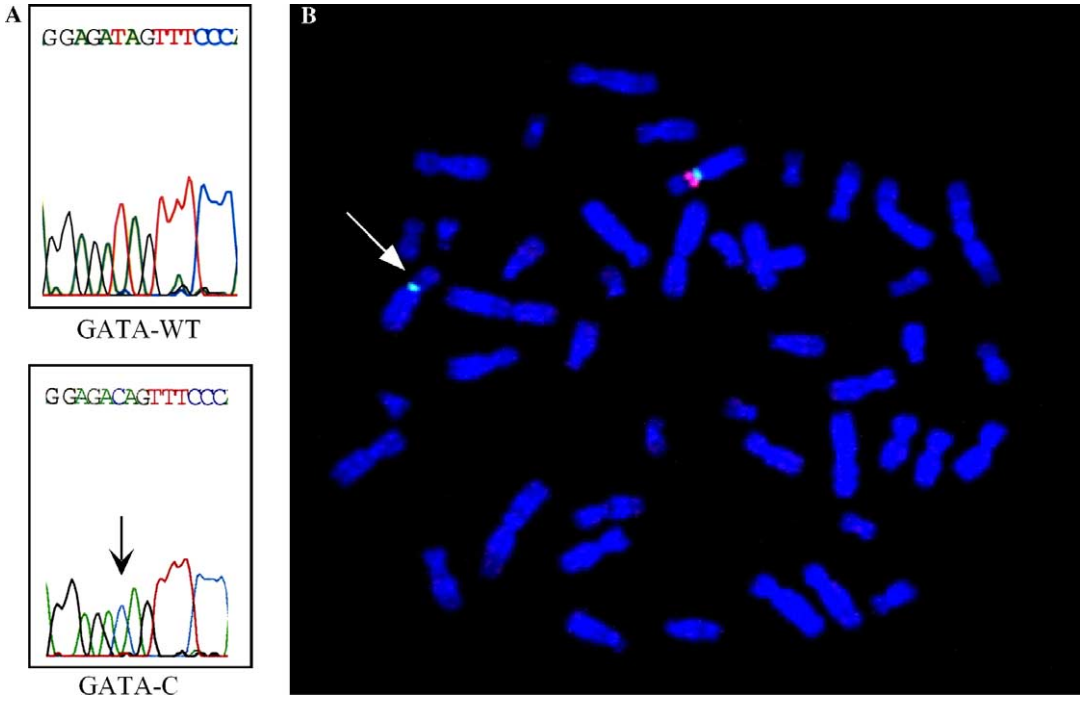

Fig. 3. Analysis of the *RUNX2* mutations in the CCD patient with the deletion of one *RUNX2* allele and the (−1176)T > C unique sequence variant in the *RUNX2* promoter. (A) Electropherograms of the sequence variants, an arrow indicates the substitution in the GATA consensus sequence. (B) FISH analysis: representative metaphase showing a deletion of one allele of the *RUNX2* gene (arrow). The chromosome 6 centromere probe fluoresces green and the RUNX2 probe fluoresces red.

derm, bone and hair development [50–53]. Except for this patient and her mother, the (−1176)C allele was detected in none of nearly 300 chromosomes (the control group combined with the CCD patients). Since only the (−1176)C allele and only one allele of −569(T)11_13 were detected in this patient, we considered the possibility of the deletion of one *RUNX2* allele. Indeed, the FISH analysis with the probe specific for *RUNX2* detected only one copy of the gene (Fig. 3B). Interestingly, in spite of the deletion of one *RUNX2* allele, bone mineral density in this patient is elevated (> + 3.3 *Z* score at the lumbar spine), different from either normal or decreased bone mineral density that we have observed in patients with CCD (data not shown).

Functional analysis of the RUNX2 promoter sequence variants

To study the functional implications of the *RUNX2* promoter sequence variants, we used ROS17/2.8 and C3H10T1/2 cell lines that differ in the levels of endogenous RUNX2. ROS17/2.8 is a rat osteosarcoma cell line with high transcriptional activity of *RUNX2* while C3H10T1/2 is a mouse multipotent mesenchymal cell line with low activity of the *RUNX2* promoter.

For the electromobility shift assay (EMSA) we used as probes, fragments of the *RUNX2* promoter containing MZF1 and GATA binding sites and the (−1048)A > C substitution. We designated the probes with the most common alleles: MZF-WT and GATA-WT, 1048-WT, and the probes with uncommon SNPs: MZF-A: (−334)C > A, MZF-T: (−330)G > T, GATA-C: (−1176)T > C and 1048-C: (−1048)A > C. The EMSA assay shows that all WT probes form specific complexes in ROS17/2.8 and C3H10T1/2 cell lines and, most significantly, demonstrates that all SNPs change the DNA–protein binding affinity (Fig. 4). All three variants of the MZF-1 binding site showed differential binding affinity. In both ROS17/2.8 and C3H10T1/2 cell lines, the MZF-A probe forms a stronger complex than the probe with the most common allele (MZF-WT), while the binding is completely abolished for the probe carrying the (−330)G > T substitution. Fig. 4A shows EMSA results only in C3H10T1/2 cells since they are identical to ROS17/2.8 for all MZF-1 probes. Similarly, since EMSA results for 1048-WT and 1048-C probes in ROS17/2.8 and C3H10T1/2 cells were identical, Fig. 4C shows results in ROS17/2.8 cells only. The 1048-WT probe forms low- and high-molecular weight specific complexes (LMW and HMW) in analyzed cell lines. Binding to the low LMW proteins is abolished by the (−1048)A > C substitution. Unexpectedly, this substitution increases binding to HMW complexes (Fig. 4C). The binding affinity of the GATA probes differs in ROS17/2.8 and C3H10T1/2 cells (Fig. 4B). Although there is no apparent difference in complex formation by

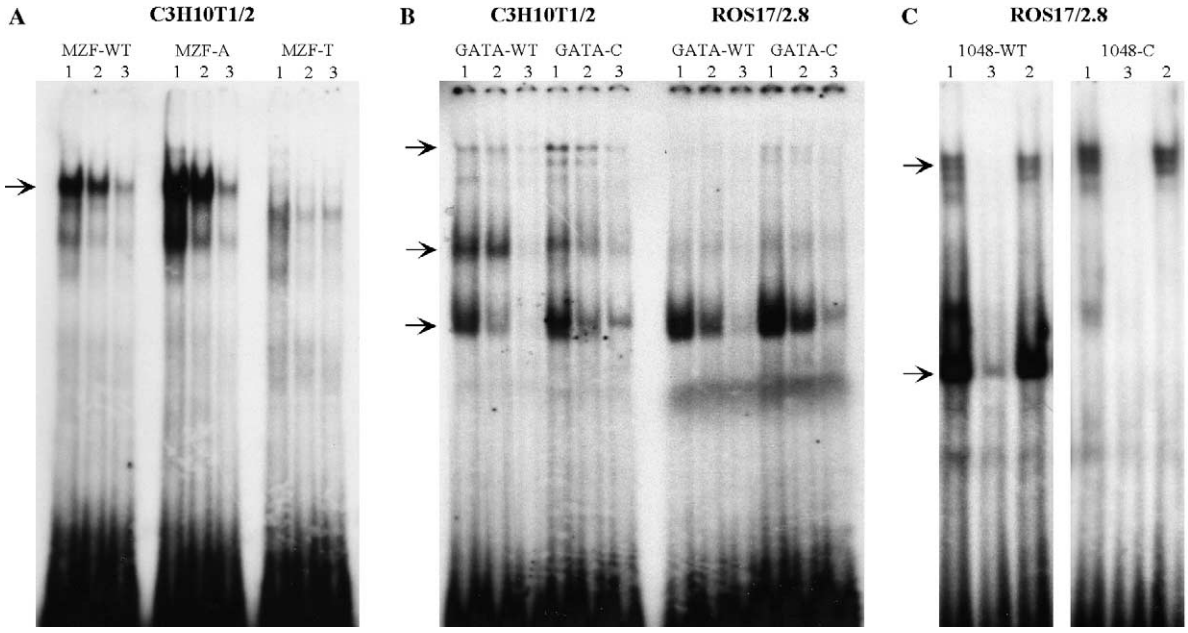

Fig. 4. Electromobility shift assay showing different binding activities of the *RUNX2* promoter sequence variants. (A) Variants located within MZF-1 binding site; (B) variant located within GATA consensus sequence; (C) variant (−1048)A > C. Lane 1, no competition; lane 2, non-specific competition; and lane 3, specific competition. Specific complexes are those that are not competed by a non-specific probe (arrows).

GATA-WT and GATA-C probes, in differentiated osteoblastic ROS17/2.8 cell line, both GATA probes form LMW complexes, whereas in multipotent C3H10T1/2 cells, there is an additional HMW complex. This difference in complex formation between the cell lines might indicate that the analyzed fragment of the *RUNX2* promoter containing the GATA consensus site might be involved in tissue specific regulation of the RUNX2 expression.

In summary, we found two promoter sequence variants that abolished formation of DNA protein complexes and one variant that increased DNA–protein interactions (Table 4).

Since three detected promoter sequence variants change the DNA–protein binding specificity in the in vitro assay, we performed expression studies in cultured cells to determine the effects of sequence variants on transcriptional activity of the *RUNX2* promoter. We generated five constructs containing the 1.3-kb fragment of the *RUNX2* promoter (the most common allele and four alleles containing the detected SNPs) driving the luciferase reporter. As expected, luciferase expression from the *RUNX2* promoter was much higher in ROS17/2.8 cells that express high levels of endogenous Runx2 than in the C3H10T1/2 cell line that does not express Runx2. While all five promoter variants have a similar transcriptional activity in multipotent C3H10T1/2 cells, the (−1048)A > C variant decreases the transcriptional activity of the *RUNX2* promoter in osteoblastic ROS17/2.8 cells (Fig. 5). For all three vector doses tested, the reporter gene expression from the (−1048)C promoter variant was approximately 30% lower than from the most common variant. The remaining three substitutions did not affect the expression of the reporter gene neither in ROS17/2.8 nor in C3H10T1/2 cell lines.

Interaction of TRPS1 with the RUNX2 promoter

The GATA-domain zinc finger transcription factors are high molecular weight proteins that have been mostly implicated in hematopoiesis. However, one family member TRPS1, is expressed during skeletal development (Napierala and Lee unpublished data, and [54]) and mutated in the human skeletal dysplasia trichorhino-phalangeal syndrome. We analyzed whether the HMW complex formed by the GATA probes contains TRPS1. For the EMSA and super-shift assays we used protein extract from COS7 cells transfected with V5-tagged TRPS1. We chose this cell line, because similar to C3H10T1/2, protein extracts from COS7 cells form a HMW complex with the GATA probes. Interestingly, the super-shift of HMW complex with anti-V5 antibodies was observed for the GATA-WT, but not the GATA-C probe (Fig. 6A). These results indicate that TRPS1 can bind the *RUNX2* promoter and the (−1176)T > C substitution disrupts the TRPS1-*RUNX2* promoter interaction.

Following the results identifying Trps1 as a high molecular weight protein that can form a complex with the GATA consensus site within the *RUNX2* promoter, we decided to analyze whether Trps1 can affect the activity of the *RUNX2* promoter. Since the Trps1- *RUNX2* promoter complex is present only in nonosteoblastic cell lines and *Trps1* acts as a transcriptional repressor [55],

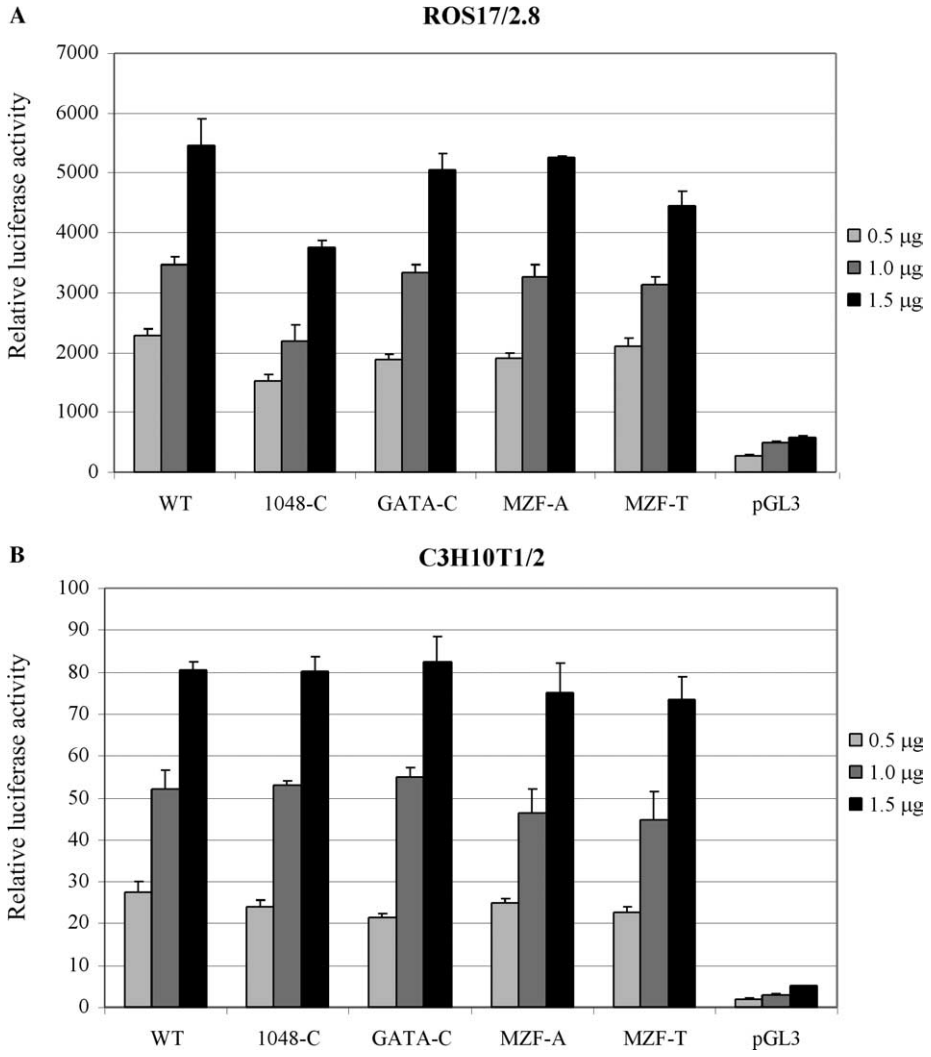

Fig. 5. Transcriptional activity of the *RUNX2* promoter variants in ROS17/2.8 (A) and C3H10T1/2 (B) cell lines. All the transfection data represent ratios of luciferase to β-galactosidase activities and values are means of three independent transfection experiments. Error bars represent standard deviation. Three different colors of bars represent different dosages of the reporter plasmid.

we hypothesized that Trps1 can inhibit the *Runx2* expression. Analyses were performed in ROS17/2.8 cells, where the *RUNX2* promoter is highly activated. Co-transfection experiments showed that the expression of the reporter from the 1.3-kb *RUNX2* promoter is reduced upon co-transfection with a TRPS1-expressing plasmid (Fig. 6B). These results suggest that *Trps1* can inhibit the activity of the *RUNX2* promoter in vitro.

Discussion

RUNX2 is the key transcription factor regulating bone formation and homeostasis [56–59]. Heterozygous nonsense mutations or missense mutations affecting activity of the RUNX2 protein cause cleidocranial dysplasia (CCD). Although the CCD phenotype has shown genetic linkage only to the *RUNX2* locus, no mutations are detected in up to one third of patients referred for DNA analysis. Approximately 100 CCD patients have been screened for mutations in the *RUNX2* coding sequence in our laboratory over last 6 years. While the initial *RUNX2* screens included only patients with classical presentation of cleidocranial dysplasia, later analysis included patients with atypical CCD phenotypes, the majority of which are sporadic cases. Far fewer mutations were observed within this group. While classic CCD is not considered a heterogeneous disease, it is possible that "CCD-like" patients carry mutations in genes upstream or downstream in the RUNX2 pathway or in *RUNX2 cis*-regulatory elements. Moreover, promoter variants might affect *Runx2* expression and either be directly pathogenic or act as modifiers of an existing mutation on the other allele.

Another mechanism accounting for CCD might include *RUNX2* promoter mutations that alter binding sites of transcription factors important for *RUNX2* expression. It has been demonstrated that many pro-

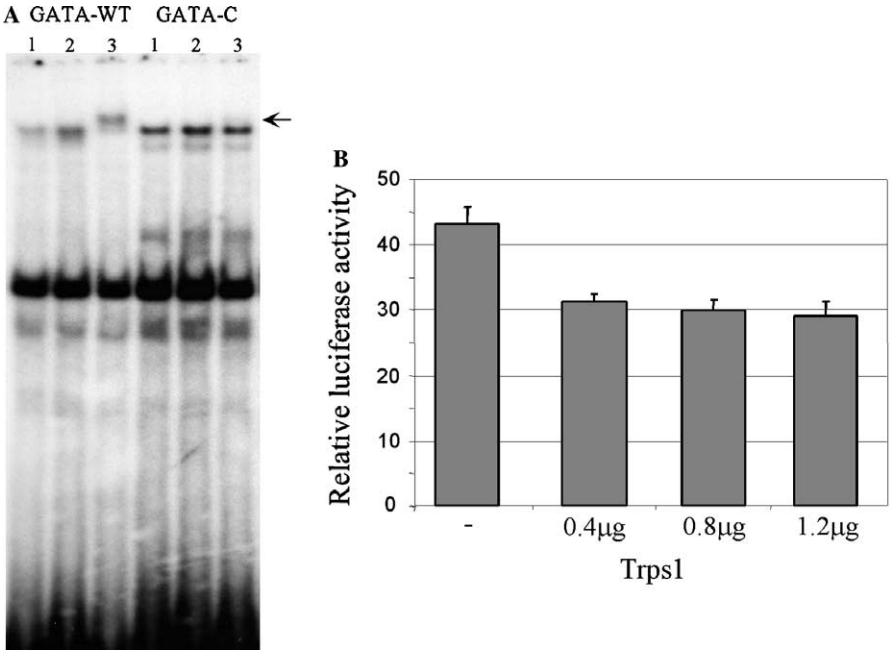

Fig. 6. Identification of TRPS1 as a potential *trans*-acting factor regulating *RUNX2* expression. (A) Electromobility shift assay and super-shift with anti-V5 antibodies. TRPS1 forms complex with GATA-WT probe, but not with mutant GATA-C probe (arrow). Lane 1, no serum; lane 2, pre-immune serum; and lane 3, anti-V5 antibodies. (B) TRPS1 represses transcriptional activity of the *RUNX2* promoter in ROS17/2.8 cell line. Doses of Trps1-expressing plasmid are indicated below the bars.

moter sequence variants can modify in vitro gene expression by as much as 50% [60]. Interestingly, specific knock-out of the P1 promoter and first 19 amino acids of Runx2 in mice revealed that they are important for complete osteoblast maturation and endochondral bone formation [42]. Therefore, we analyzed the *RUNX2* promoter in CCD patients without detectable mutations in the *RUNX2* coding region to investigate whether changes in the promoter sequence could lead to haploinsufficiency or partial loss of function. To our knowledge, this is the first detailed analysis of the *RUNX2* promoter in CCD patients. We found four single nucleotide changes in the *RUNX2* promoter in CCD patients and controls. Although population studies and functional analysis do not indicate that these promoter variants cause the CCD phenotype, they might potentially modify *RUNX2* expression. In support for this, all four promoter variants exhibit specific DNA–protein interactions as demonstrated by electromobility shift assay. These in vitro results raise the question of whether different haplotype combinations of these promoter SNPs might affect the transcriptional activity of RUNX2 and contribute to the *RUNX2* phenotypic spectrum.

We found two SNPs that might be clinically relevant. In family 102-43, the mother harbors two variants: one that increases protein binding (−334A allele) and one that does not form a complex (−330T allele). Whether this compound heterozygosity prevents clinical symptoms is unknown. The affected child has only the (−334)C > A substitution without other RUNX2 mutation. It could be speculated that the absence of the higher affinity allele has uncovered the phenotype in the daughter. Also mutation in a yet unidentified *trans* factor in concert with the detected (−334)C > A and (−330)G > T variants might modify the phenotype. Similarly, the (−1176)T > C substitution in the GATA consensus sequence might modify the disease phenotype of the CCD patient with the deletion of one *RUNX2* allele. The EMSA analysis demonstrated that only the GATA-WT probe could form a specific high molecular weight complex with GATA-type transcriptional repressor TRPS1 and that the (−1176)T > C substitution abolished this binding. In vivo, loss of repression of the promoter due to mutation in one of the GATA consensus sequences might cause increased expression of *RUNX2*. This might contribute to increased bone mineral density observed in this patient in spite of the deletion of the other *RUNX2* allele. Additionally, presence of a TRPS1 complex in the non-osteoblastic cells, but not in osteoblastic cells raises the question of whether *Trps1* might specify the tissue specific expression of *Runx2* by negatively regulating its expression in cells not destined for osteoblastic lineages. In fact, *Trps1* is highly expressed in mesenchymal condensations destined for chondrogenic lineages. In co-transfection experiment we showed that Trps1 can repress the *RUNX2* promoter. Lack of apparent response of the *RUNX2* promoter to the dose of Trps1 expressing plasmid in ROS17/2.8 cells suggests that

additional tissue specific factors might be involved in Trps1 repression activity in cells where Runx2 needs to be turned off. In fact, EMSA experiments demonstrated that DNA probes with GATA consensus sequence form more than one specific complex and one of these complexes is absent in ROS17/2.8 cells where Runx2 is highly expressed. Other proteins that bind to the fragment of the RUNX2 promoter containing GATA consensus site remind to be determined.

Our in vitro studies showed that some *RUNX2* promoter variants are associated with alterations in transcriptional activity of the *RUNX2* promoter (Table 4). Moreover, they identify DNA–protein complexes that might contain transcription factors that specify the spatial and temporal control of *RUNX2* expression. Analysis of the promoter SNPs identified the TRPS1 transcriptional repressor as a new potential regulator of *Runx2* expression. Polymorphic variants of proteins regulating the *RUNX2* expression, in conjunction with the detected alterations of the *RUNX2* promoter might act as modifiers of quantitative traits such as bone mineral density [61,62]. The results of our studies may aid in the identification of this determinants of complex bone disorders.

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