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Report

Dysregulation of Chondrogenesis in Human Cleidocranial Dysplasia

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Cleidocranial dysplasia (CCD) is an autosomal dominant skeletal dysplasia caused by heterozygosity of mutations in human *RUNX2*. The disorder is characterized by delayed closure of the fontanel and hypoplastic clavicles that result from defective intramembranous ossification. However, additional features, such as short stature and cone epiphyses, also suggest an underlying defect in endochondral ossification. Here, we report observations of growth-plate abnormalities in a patient with a novel *RUNX2* gene mutation, a single C insertion (1228insC), which is predicted to lead to a premature termination codon and thus to haploinsufficiency of *RUNX2* and the CCD phenotype. Histological analysis of the rib and long-bone cartilages showed a markedly diminished zone of hypertrophy. Quantitative real-time reverse transcription–polymerase chain reaction analysis of limb cartilage RNA showed a 5–10-fold decrease in the hypertrophic chondrocyte molecular markers *VEGF*, *MMP13*, and *COL10A1*. Together, these data show that humans with CCD have altered endochondral ossification due to altered *RUNX2* regulation of hypertrophic chondrocyte–specific genes during chondrocyte maturation.

Runx2 is a well-characterized critical transcriptional regulator of osteoblast differentiation (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997). Heterozygosity for mutations in *RUNX2* causes cleidocranial dysplasia (CCD [MIM 119600]), an autosomal dominant skeletal dysplasia characterized by hypoplastic clavicles, short stature, and tooth anomalies (Lee et al. 1997; Mundlos et al. 1997; Lee and Zhou 2004). *Runx2*-deficient mice (−/−) have no bone formation, whereas the heterozygote mice (+/−) display abnormalities similar to those seen in individuals with CCD (Shapiro 1999). *Runx2* heterozygote mice have fewer osteoblasts and bone-specific proteins, and the enzyme activity of alkaline phosphatase is low (Shapiro 1999). Furthermore, molecular and mouse genetic studies demonstrate that the abnormal bone formation is mainly due to *Runx2* transcriptional regulation of its target genes during osteoblast differentiation. In osteoblasts, transcription of the bone-specific osteocalcin gene is principally regulated by the *Runx2* transcription factor and is stimulated in response to vitamin D_3 via the vitamin D_3 receptor complex (Sierra et al. 2003). Beyond the skeleton, studies suggest that *Runx2* regulates the expression of molecules in mesenchyme that act reciprocally on the dental epithelium to control its growth and differentiation, and this partly explains the dental abnormalities found in patients with CCD and in *Runx2* heterozygote mice (D'Souza et al. 1999; Zou et al. 2003).

The CCD characteristics of delayed closure of the fontanel and hypoplastic clavicles reflect defective intramembranous ossification. However, the significantly hypoplastic or absent clavicles point to an effect on both intramembranous and endochondral ossification, because the clavicle ossifies embryologically by both routes (Huang et al. 1997). Recent reports that emphasize short stature as a prominent feature in CCD provide supporting evidence for *RUNX2* effects on endochondral ossification (Cooper et al. 2001; Morava et al. 2002). In addition, mouse genetics studies have shown altered chondrocyte maturation or defective chondrocyte hypertrophy in some long bones of *Runx2* null mice (Inada et al. 1999; Enomoto et al. 2000). Growth-plate compression and decreased expression of *Col10a1* (a hy-

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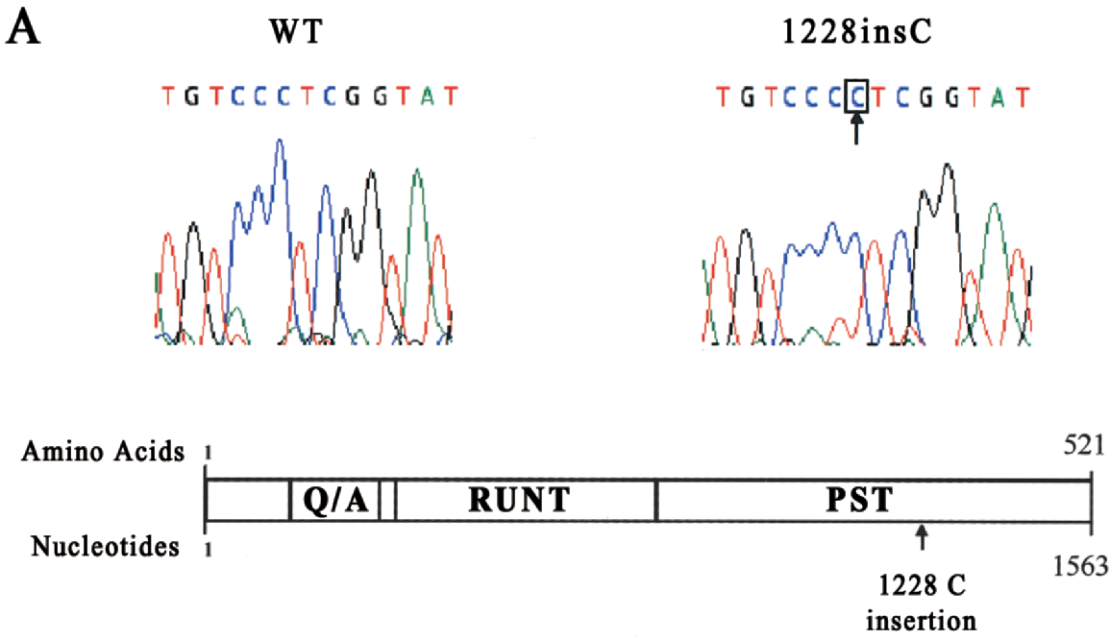
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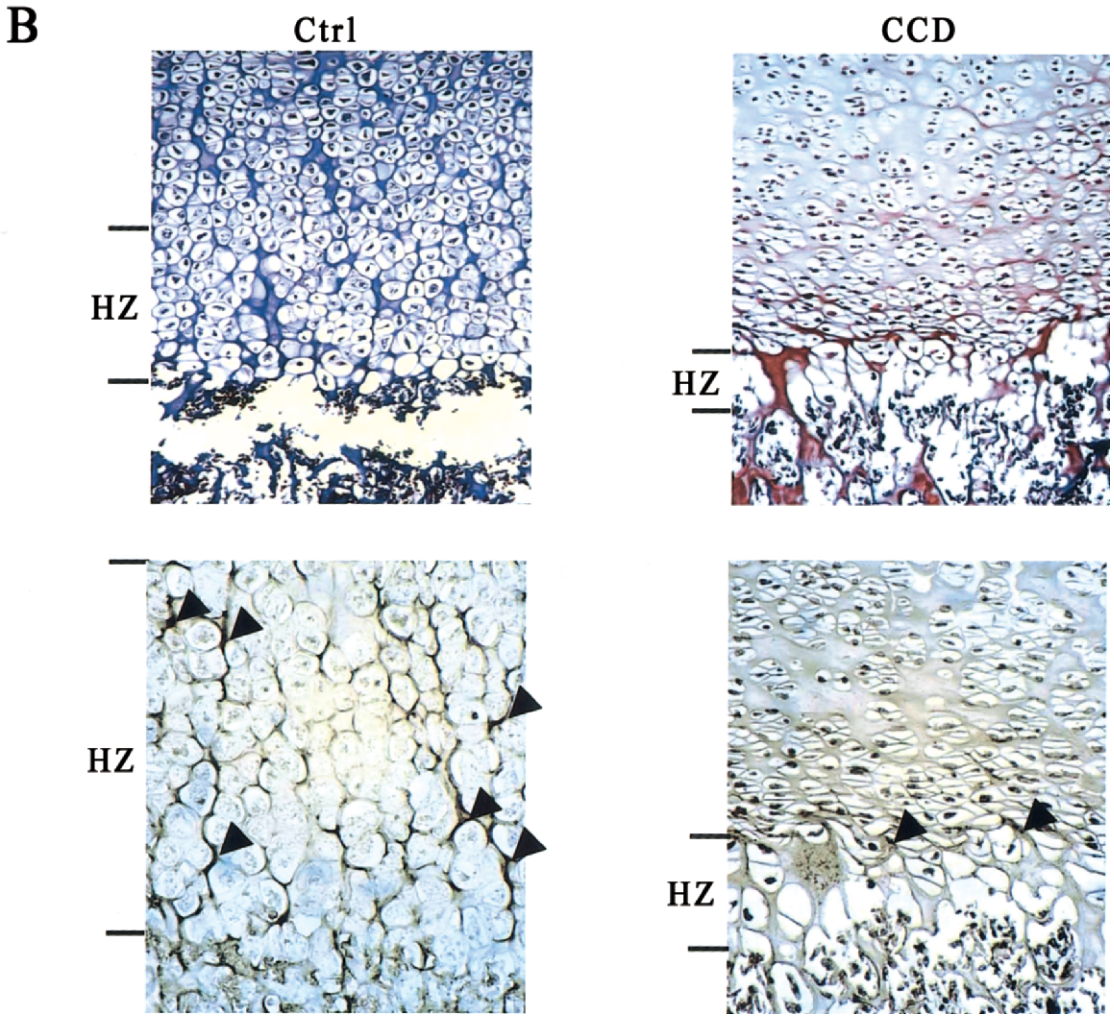
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pertrophic chondrocyte–specific molecular marker) have also been reported in *Runx2* heterozygote mice (Zheng et al. 2003). However, the molecular and histological correlates in human development have only been extrapolated.

In this study, we report the molecular, immunohistochemical, and histological characterization of growth-plate abnormalities in a 20-wk fetus with CCD and a novel *RUNX2* gene mutation. Prenatal diagnosis of a de novo case of CCD was made by ultrasonography at 19 wk. On the basis of ultrasound long-bone measurements, the 20-wk fetus was smaller than normal, with limb lengths consistent with 19-wk gestation, and absent clavicles and a hypomineralized calvarium were noted. Relevant tissues, including the entire distal femoral cartilage, were harvested at autopsy. *RUNX2* exons were PCR amplified from genomic DNA by use of intron-specific primers, as described elsewhere (Lee et al. 1997). Direct sequencing of this PCR-amplified *RUNX2* gene revealed a single C insertion (1228insC) resulting in a frameshift mutation in codon 410 (fig. 1*A*). The mutation was confirmed by cloning and sequencing of each allele. This mutation is predicted to lead to a premature termination codon at amino acid 489 in the proline, serine, and threonine–rich (PST) transactivation domain and to alteration of the identity of the last 112 amino acids in the peptide (fig. 1*A*). On the basis of previous work, this mutation is predicted to cause haploinsufficiency and to produce the CCD phenotype (Lee et al. 1997; Mundlos et al. 1997; Zhou et al. 1999). Interestingly, one nearby mutation (1205insC), which leads to a frameshift in codon 402, was reported to cause a severe CCD phenotype, with osteoporosis, recurrent bone fractures, and scoliosis (Quack et al. 1999). Another adjacent R391X premature termination mutation was previously reported to be associated with classic CCD; in an in vitro transfection study, this mutated protein showed only 20% transactivation of the reporter activity, compared with wild-type Runx2 (Zhou et al. 1999).

To study the putative skeletal deficiency in this fetus with CCD, we performed histologic analysis of rib and other long-bone cartilage from the fetus and an age-matched control. The rib, tibia, and femur from the fetus with CCD and the control were formalin-fixed and embedded in paraffin. Von Kossa staining was performed on 5-μm sagittal sections (Lowe and Bancroft 1996). The rib growth-plate sections of the fetus with CCD showed a markedly diminished zone of hypertrophy and disorganized growth-plate chondrocytes, compared with those of the age-matched control (fig. 1*B, upper panel*). Similar findings were also found for the ulna and tibia (data not shown). Because of the significant shortened zone of hypertrophy, we performed immunohistochemical analysis with both rib and limb sections, using an antibody specific to type X collagen (from Dr. Robin Pool, Montreal), a molecular marker of chondrocyte hypertrophy during endochondral ossification. The result showed altered type X collagen expression and localization. In the control growth plate, type X collagen staining was seen primarily in the pericellular matrix. In contrast, in the CCD growth plate, the type X collagen staining was poorly visible in the extracellular matrix and was more localized to intracellular space (fig. 1*B, lower panel,* and figs. 2 and 3). These data together suggest an important function for *RUNX2* in human endochondral ossification, linking haploinsufficiency of *RUNX2,* decreased chondrocyte hypertrophy, and altered type X collagen expression and distribution in CCD.

Runx2 has been shown to be an important regulator of chondrocyte maturation, by in vitro cell culture and in vivo mouse genetics studies (Inada et al. 1999; Kim et al. 1999; Enomoto et al. 2000; Takeda et al. 2001; Ueta et al. 2001). In *Runx2*-deficient mice (−/−), chondrocyte differentiation was reported to be severely disturbed (Inada et al. 1999), and lack of hypertrophic chondrocytes with delayed onset of hypertrophy has also

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics.*

Figure 2 Hematoxylin and eosin staining and immunohistochemical analysis of the CCD and control femur sections. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics.*

Figure 1 Molecular and histological analyses of a fetus with CCD. *A,* Sequencing of exon 9 of *RUNX2* alleles shows the mutant allele with a C insertion in the PST domain (1228insC) and the wild-type allele (WT). The relative position of this mutation is shown in the schematic representation of the RUNX2 structure. Q/A = polyglutamine/polyalanine domain; RUNT = DNA-binding runt domain. *B,* Von Kossa staining and immunohistochemical analysis of the CCD rib cartilage. For an age-matched control fetal rib section (Ctrl), Von Kossa histological analysis shows a normal hypertrophic zone (*top left panel*), and type X collagen immunostaining exhibits normal extracellular staining of type X collagen in the hypertrophic zone (*arrowheads in bottom left panel*). Separation of primary spongiosa is an artifact of tissue preparation. For the rib section from the fetus with CCD, Von Kossa staining shows a much shortened hypertrophic zone (*top right panel*), and type X collagen immunostaining shows flattened hypertrophic chondrocytes, with both reduced and asymmetric type X collagen expression within the compressed hypertrophic zone (*arrowheads in bottom right panel*). HZ = hypertrophic zone of growth plate.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics.*

Figure 3 Von Kossa staining and immunohistochemical analysis of the CCD and control rib cartilage sections. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics.*

been observed (Kim et al. 1999). Although type X collagen was barely detectable in *Runx2* null mice, osteopontin, bone sialoprotein, and collagenase 3 (MMP-13) were also not expressed in hypertrophic chondrocytes, indicating that they are regulated by *Runx2* in terminal hypertrophic chondrocytes (Inada et al. 1999). In recent studies, this has been shown to be the result of direct *Runx2* regulation of their respective promoters (Sato et al. 1998; Jimenez et al. 1999; Zheng et al. 2003; Wang et al. 2004). Vascular endothelial growth factor (VEGF), a critical regulator of angiogenesis during development, is another direct transcriptional target of *Runx2*. *Runx2* is required for a tissue-specific genetic program that regulates VEGF during endochondral bone formation (Zelzer et al. 2001). More recently, mouse genetics studies have pointed out the possible cooperative function of Runt gene family members—*Runx1, Runx2,* and *Runx3*—in skeletal development. *Runx1* mediates early events of endochondral and intramembranous bone formation, *Runx3* cooperates with *Runx2* in promoting chondrocyte maturation, and *Runx2* is a potent inducer of late stages of chondrocyte and osteoblast differentiation (Yoshida et al. 2004; Smith et al. 2005).

To further characterize the molecular mechanisms and signaling pathways that are involved in the pathogenesis of CCD in humans, we performed quantitative real-time RT-PCR analysis of growth-plate RNA, to determine the status of the *RUNX2* transcriptional network in cartilage. We performed assays for the relative expression levels of known *RUNX2* targets *VEGF, MMP13,* and *COL10A1,* as well as for the transcription factor genes *RUNX1, RUNX2, RUNX3,* and *SOX9*. Total RNAs from CCD and age-matched control cartilages (from bilateral distal femurs) were extracted as described elsewhere (Zheng et al. 2003). cDNA synthesis from the total RNA, real-time PCR amplification of target genes, and analysis of the real-time PCR results for fold induction of gene expression were performed in accordance with the manufacturer's protocol and as in previously reported procedures with modifications (Livak and Schmittgen 2001; Pfaffl 2001; Zheng et al. 2003). The sequences of the primers used for PCR amplification of *RUNX1, RUNX2, RUNX3, COL10A1, MMP13, SOX9, VEGF,* and *GAPDH* are listed in table 1. The *GAPDH* gene was used as an internal control for the quantity and quality of the cDNAs.

Our results showed that the level of *RUNX2* mRNA was ~50% downregulated in the CCD cartilage, as compared with the control cartilage, suggesting that haploinsufficiency of *RUNX2* results from the frameshift mutation described above (figs. 1*A* and 4*A*). This mutation, which sits in the last exon of *RUNX2,* is predicted to lead to a premature termination codon (PTC). However, nonsense-mediated mRNA decay (NMD) cannot be excluded for this mutant allele (Maquat 2004). It has been reported that a PTC mutation leading to complete NMD causes collagen X haploinsufficiency in Schmid metaphyseal chondrodysplasia cartilage (Bateman et al. 2003). However, since this mutation resides in the last exon, a potential translated mRNA that escapes NMD is possible, although the production of a truncated protein has never been demonstrated for this condition. Interestingly, sequencing of 10 independent RT-PCR cDNAs showed that 3 of 10 contain the mutant allele, which suggests the possibility that the transcript escapes NMD. We also examined the hypertrophic chondrocyte–related molecular markers *COL10A1, MMP-13,* and *VEGF,* and they all showed a 5–10-fold decrease in mRNA levels (fig. 4*B,* 4*C,* and 4*D*). These genes are highly expressed in terminally differentiated chondrocytes. Because of the significantly shortened zone of hypertrophy, we dissected the whole growth-plate cartilages to examine the expression level of the specific genes mentioned above, with normalization to the house-keeping gene *GAPDH*. Although the reduced expression of target genes might be partially due to the reduced number of hypertrophic chondrocytes, these data generated in human tissue correlate well with the in vitro and in vivo data for mice showing the direct regulation of the respective target promoters by *Runx2* during skeletal development (Jimenez et al. 1999; Zelzer et al. 2001; D'Alonzo et al. 2002; Zheng et al. 2003). The observation of a shortened zone of hypertrophy was previously reported for *Runx2* heterozygote mice and also correlates well with human data (Zheng et al. 2003). No change in mRNA levels was observed for *RUNX1* (fig. 4*E*). Interestingly, we also examined the *RUNX3* mRNA level by real-time RT-PCR analysis, and it showed a 4-fold upregulation in CCD cartilage, compared with control cartilage (fig. 4*F*). This raises the question of whether *RUNX3* expression may be upregulated in compensation for loss of *RUNX2*. Recent

Table 1

Sequences of Primers Used for PCR Amplification

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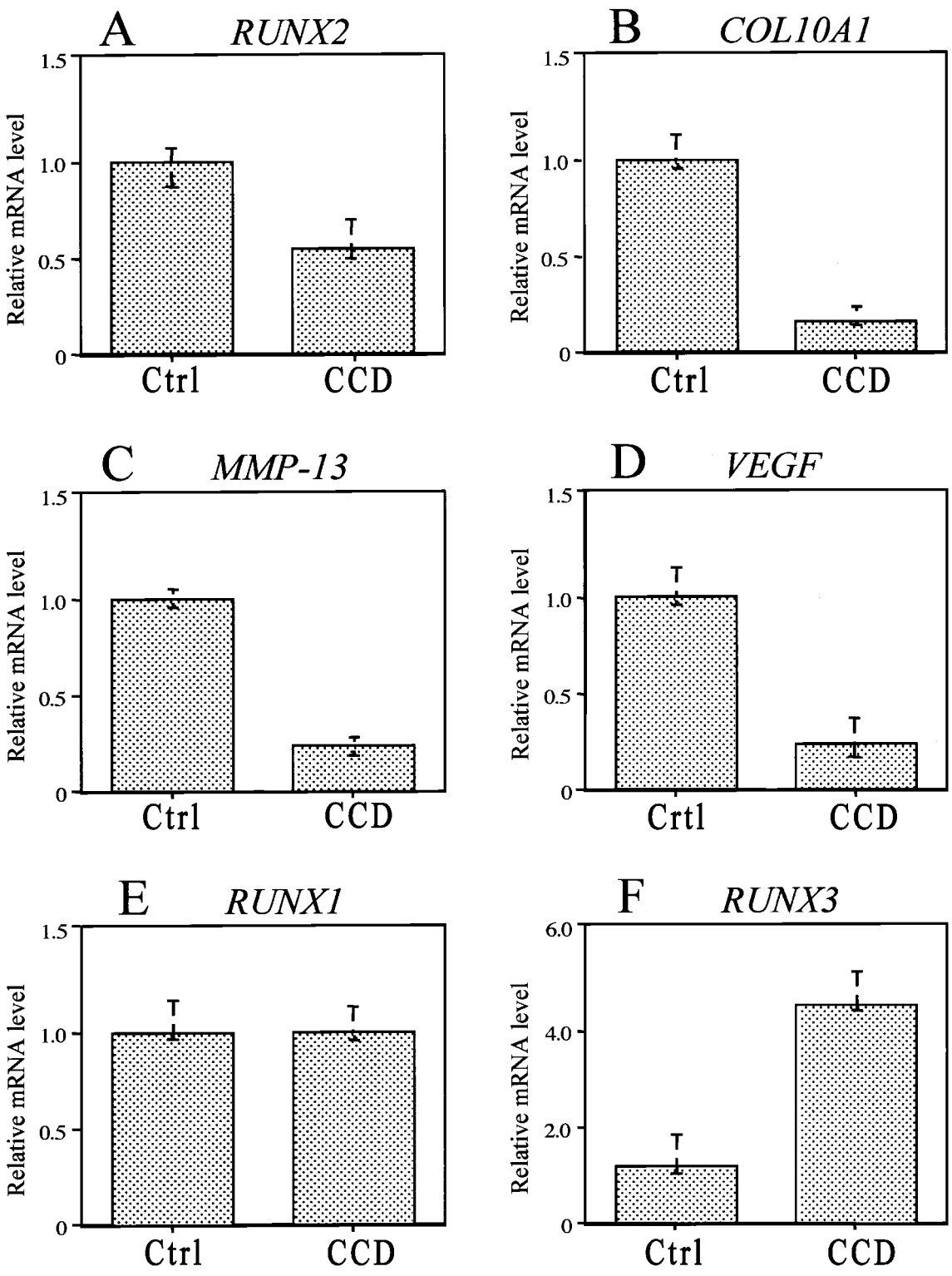

Figure 4 Real-time RT-PCR analysis of genes expressed in limb cartilage. Amplification was performed on a LightCycler (Fast Start DNA Master SYBR Green I [Roche Diagnostics]). Analysis of the results (i.e., the relative gene expression level) was achieved by normalization to *GAPDH* and by comparison with the age-matched control for fold induction. *A,* The mRNA level of *RUNX2* is decreased by 50% in the fetus with CCD, compared with the control. *B, C,* and *D,* The hypertrophic chondrocyte–related molecular markers *COL10A1, MMP-13,* and *VEGF* showed 10-, 7.6-, and 8-fold decreases in mRNA levels, respectively. *E,* No change in mRNA level was observed for *RUNX1. F, RUNX3* showed 4-fold up-regulation in fetus with CCD, compared with the age-matched control. Each real-time RT-PCR experiment was performed in triplicate, and one representative set of results is presented here, with SDs shown by error bars. Ctrl = age-matched control.

mouse genetics studies suggest that both *Runx2* and *Runx3* are essential for chondrocyte maturation and that the delayed chondrocyte maturation in *Runx2* heterozygotes on a *Runx3* null background demonstrated that *Runx3* is required for endochondral ossification when the *Runx2* gene dosage is halved (Yoshida et al. 2004; Komori 2005). No mRNA level change was observed for *SOX9*, a transcription factor required for chondrogenesis and a regulator of the type II collagen gene (Lefebvre et al. 1998). This supports the selective defect present in prehypertrophic and hypertrophic chondrocytes and not in the proliferating and resting chondrocytes of the growth plate in this fetus with CCD.

CCD is caused by haploinsufficiency of *RUNX2*, and no significant phenotypic differences have been observed between patients with deletions or frameshifts and patients with missense mutations of *RUNX2* (Quack et al. 1999). There is a correlation between phenotypic short stature and tooth anomalies in some patients with CCD (Yoshida et al. 2003). Unlike many skeletal dysplasias that primarily affect cartilage, CCD affects both cartilage and bone (Mundlos 1999; Hermanns and Lee 2001; Lee and Zhou 2004). Since *RUNX2* is required for both osteoblast differentiation and chondrocyte maturation, it links chondrocyte and osteoblast differentiation during endochondral bone formation (Mundlos 1999; de Crombrugghe et al. 2001). Many other transcription factors, such as the Sox family members, and signaling molecules, including Indian hedgehog, parathyroid hormone, and related peptide (PTH/PTHrP) and fibroblast growth factors, also link chondrocyte and osteoblast differentiation pathways during endochondral bone formation (Lanske et al. 1996; Vortkamp et al. 1996; Schipani et al. 1997; St-Jacques et al. 1999; Chung et al. 2001; de Crombrugghe et al. 2001). Not surprisingly, some of these pathways have been shown to impinge on Runx2 activity. Our studies demonstrate altered chondrocyte hypertrophy in human CCD, with decreased expression of hypertrophic chondrocyte–related markers that have been shown to be transcriptional targets of *Runx2* in mouse genetics studies (Jimenez et al. 1999; Zelzer et al. 2001; Wong et al. 2003; Zheng et al. 2003; Varghese et al. 2005). On the basis of these data and observations in mice, we can conclude that abnormal endochondral ossification in CCD results from altered *RUNX2* regulation of chondrocyte hypertrophy and is associated with downregulation of important *RUNX2* transcriptional targets, including *COL10A1, VEGF*, and *MMP13*.

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Web Resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CCD)

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