Dicer-dependent pathways regulate chondrocyte proliferation and differentiation

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Edited by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, TX, and approved December 18, 2007 (received for review August 22, 2007)

Small noncoding RNAs, microRNAs (miRNAs), bind to messenger RNAs through base pairing to suppress gene expression. Despite accumulating evidence that miRNAs play critical roles in various biological processes across diverse organisms, their roles in mammalian skeletal development have not been demonstrated. Here, we show that Dicer, an essential component for biogenesis of miRNAs, is essential for normal skeletal development. *Dicer*-null growth plates show a progressive reduction in the proliferating pool of chondrocytes, leading to severe skeletal growth defects and premature death of mice. The reduction of proliferating chondrocytes in *Dicer*-null growth plates is caused by two distinct mechanisms: decreased chondrocyte proliferation and accelerated differentiation into postmitotic hypertrophic chondrocytes. These defects appear to be caused by mechanisms downstream or independent of the *Ihh*-PTHrP signaling pathway, a pivotal signaling system that regulates chondrocyte proliferation and differentiation. Microarray analysis of *Dicer*-null chondrocytes showed limited expression changes in miRNA-target genes, suggesting that, in the majority of cases, chondrocytic miRNAs do not directly regulate target RNA abundance. Our results demonstrate the critical role of the Dicer-dependent pathway in the regulation of chondrocyte proliferation and differentiation during skeletal development.

microRNA | skeletal development

Endochondral bone development is composed of the initial formation of a cartilage template and its subsequent replacement by mineralized bone. Longitudinal bone growth is driven by regulated proliferation and differentiation of chondrocytes in the growth plate cartilage. In developing growth plates, periaricular chondrocytes proliferate and differentiate into flat columnar chondrocytes that proliferate further to form orderly columns. Columnar chondrocytes stop proliferating and then differentiate into postmitotic hypertrophic chondrocytes. This process is tightly controlled by multiple layers of regulatory mechanisms, thus allowing persistent longitudinal bone growth.

Regulation of gene expression is the major mechanism to control a variety of cellular functions, including proliferation and differentiation. Small noncoding microRNAs (miRNAs) encoded in the genome regulate gene expression at the posttranscriptional level. Genetic ablation of miRNA genes has demonstrated that loss of single miRNAs can result in significant physiological consequences in mice (1–4). In addition, germ-line ablation of genes encoding components for miRNA biogenesis results in embryonic or perinatal lethality in mice (5–7). These examples suggest that posttranscriptional gene regulation by miRNAs plays a critical role in regulating fundamental cellular functions in mice. miRNAs are generated from long primary transcripts (pri-miRNAs) through multiple processing steps (8). pri-miRNAs are cleaved into small-hairpin pre-miRNAs by the microprocessor complex containing Drosha and DGCR8. pre-miRNAs are exported into the cytoplasm, where the RNase III, Dicer, removes the loop region of the hairpin. This step is

essential for generation of mature miRNAs. To investigate the role of Dicer-dependent small RNAs in skeletal development, we conditionally disrupted the *Dicer* gene in this study. We show that Dicer plays a critical role in maintaining the proliferating pool of chondrocytes through regulation of chondrocyte proliferation and inhibition of premature differentiation to postmitotic hypertrophic chondrocytes.

Results

Dicer Deficiency in Chondrocytes Causes Defects in Skeletal Development. To investigate the physiological role of Dicer-dependent small RNAs in skeletal development, we deleted the *Dicer* gene in cartilage by crossing mice containing a floxed *Dicer* allele (*Dicer*^{fl/fl}) (9) with transgenic mice expressing Cre recombinase under the control of a *Col2a1* promoter (*Col2-Cre*). This *Cre* allele exhibited efficient Cre-recombinase activity in chondrocytes (10). *Col2-Cre:Dicer*^{fl/fl} mice showed a significant growth defect and mostly die by the time of weaning. Skeletal preparation demonstrated relatively proportional reduction in skeletal size (Fig. 1A). However, the growth defect of the skull and maxilla in *Col2-Cre:Dicer*^{fl/fl} mice caused relative overgrowth of the mandible and lower incisors (Fig. 1B). This is likely due to growth defects in the nasal cartilage, because we observed histological changes similar to those of limb growth plates (data not shown). Histological examination of postnatal 12-day-old (P12) mice shows a delay in formation of the secondary ossification center and flattening of the tibial epiphysis of *Col2-Cre:Dicer*^{fl/fl} mice (Fig. 1C). The chondrocyte density in *Col2-Cre:Dicer*^{fl/fl} growth plates was reduced because of the reduction in the number of columnar proliferating chondrocytes ($1,656 \pm 49/\text{mm}^3$ vs. control $2,266 \pm 108/\text{mm}^3$; $P < 0.05$ by ANOVA, $n = 3$ per each group) (Fig. 1D). To assess the efficiency of *Dicer* removal, we quantified the mRNA level of *Dicer* mRNA by quantitative RT-PCR (qRT-PCR) using RNA isolated from microdissected hindlimb cartilage of 3-day-old mice. The *Dicer* mRNA level of *Col2-Cre:Dicer*^{fl/fl} cartilage was reduced by 87% of that of control cartilage (Fig. 1E). As expected, the expression levels of ubiquitous miRNAs, let-7a, and miR-30c were significantly reduced in *Col2-Cre:Dicer*^{fl/fl} mice (Fig. 1F).

Analysis of fetal and neonatal *Col2-Cre:Dicer*^{fl/fl} bones revealed a reduction in bone width and an expansion of the hypertrophic region of the growth plate (Fig. 2A and B). The

Author contributions: T.K. designed research; T.K. and J.L. performed research; T.K., B.S.C., S.J.R., A.P.M., E.S., M.M., and H.M.K. contributed new reagents/analytic tools; T.K. analyzed data; and T.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0707900105/DC1.

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Fig. 1. Dicer is required for normal skeletal development. (A) Skeletal preparation of 9-day-old mice. *Col2-Cre:Dicer^{fl/fl}* mice (cKO) show postnatal growth retardation compared with control littermates (Ctrl). (B) cKO mice at postnatal day 17 show growth defect of the maxilla, causing a relative overgrowth of the mandible. (C and D) Hematoxylin/eosin-stained 12-day-old tibiae. The epiphysis (brackets) of cKO mice is flattened, and formation of the secondary ossification center is delayed (C). The number of column-forming proliferating chondrocytes (brackets) in cKO mice is reduced (D). (E) qRT-PCR analysis using RNA isolated from microdissected hindlimb cartilage of 3-day-old mice shows a reduction in *Dicer* mRNA level by 87% in cKO mice. (F) Northern blot analysis on RNA of hindlimb cartilage of 3-day-old mice shows reduced expression of *let-7b* and *miR-30c* in cKO mice.

reduction in bone width was already evident at embryonic day (E)14.5, and the expansion of the hypertrophic region was present after the initiation of bone marrow formation at E15.5 (data not shown). At E18.5, the hypertrophic region, indicated by *Col10a1* expression, was expanded, whereas the *Mmp13* expression domain of the cartilage was not expanded in *Col2-Cre:Dicer^{fl/fl}* mice (Fig. 2B and C). Because *Mmp13* is expressed only by terminally differentiated hypertrophic chondrocytes in the growth plate, these observations suggest that the expansion of the hypertrophic region was caused by an acceleration of hypertrophic differentiation of proliferating chondrocytes rather than a reduction in cartilage resorption by bone cells, which would cause an increase in terminally differentiated hypertrophic chondrocytes. Expansion of the hypertrophic region can also occur as a consequence of stimulation of chondrocyte differentiation at earlier steps (11, 12). To test whether *Dicer* deficiency directly stimulated hypertrophic differentiation of proliferating chondrocytes, we deleted *Dicer* only in late proliferating chondrocytes using *Osx-Cre* transgenic mice (13). *Osx-Cre* mice show Cre recombination activity in late-proliferating chondrocytes of the growth plate and in osteoblasts (Fig. 2D). *Osx-Cre:Dicer^{fl/fl}* mice showed an expansion of hypertrophic region without affecting the size of the periarticular region (Fig. 2E). We also observed increased *Col10a1* expression in primary rib chondrocytes upon *Dicer* deletion *in vitro* [supporting information (SI) Fig. 6]. These observations strongly suggest that the loss of *Dicer* in proliferating chondrocytes directly stimulates their hypertrophic differentiation, leading to the expansion of the hypertrophic region during fetal and neonatal stages.

Fig. 2. Acceleration of hypertrophic differentiation of *Dicer*-null chondrocytes. (A) The bone width (double arrows) is reduced, and the hypertrophic region of the growth plate is expanded in neonatal *Col2-Cre:Dicer^{fl/fl}* (cKO) mice. (B) *In situ* hybridization analysis demonstrates the expansion of the hypertrophic region in E18.5 cKO growth plates of the radius, as indicated by the enlargement of the *Col10a1* domain, whereas there is no expansion of the *Mmp13* domain that marks terminally differentiated hypertrophic chondrocytes in the growth plate (arrowheads), suggesting that acceleration of hypertrophic differentiation, rather than reduction of cartilage resorption, is responsible for the expansion of the hypertrophic region. *Mmp13* is also expressed in osteoblasts in the bone marrow. Expression of mRNAs for Indian hedgehog (*Ihh*) and its transcriptional target, *Ptch1* is preserved. (C) The longitudinal length of the *Col10a1* domain was significantly increased in cKO mice by 35% (ANOVA; $n = 3$ $P < 0.05$), whereas the *Mmp13* domain was not expanded in the cKO growth plate. (D and E) Deletion of *Dicer* in late-proliferating chondrocytes using *Osx-Cre* mice results in expansion of the hypertrophic region. Cre activity of *Osx-Cre* transgenic mice, assessed by R26R reporter assay (54), is observed in late proliferating chondrocytes in addition to cells in the osteoblast lineage (C). Neonatal *Osx-Cre:Dicer^{fl/fl}* mice (cKO) show expansion of hypertrophic region marked with *Col10a1* (D).

Indian hedgehog (*Ihh*) regulates chondrocyte differentiation and proliferation through PTHrP-dependent and -independent pathways (12, 14). The expression of *Ihh* and the transcriptional target of *Ihh* signaling, *Patched* (*Ptch1*), was preserved in *Col2-Cre:Dicer^{fl/fl}* mice, suggesting that the acceleration of hypertrophic differentiation was caused by defects either downstream or independent of *Ihh* signaling. Another critical signaling system downstream of *Ihh* that negatively regulates hypertrophic differentiation is the PTHrP signaling pathway. Because the basal expression level of PTHrP in cartilage was too low to reliably detect its possible down-regulation in *Col2-Cre:Dicer^{fl/fl}* growth plates, we took advantage of transgenic mice expressing a constitutively active PTHrP receptor (*Col2-caPPR*) to test whether possible impairment of PTHrP signaling was involved in the accelerated hypertrophic differentiation in *Col2-Cre:Dicer^{fl/fl}* mice. *Col2-caPPR* transgenic mice were able to successfully rescue growth plate abnormalities caused by loss or impairment of PTHrP signaling *in vivo* (11, 15, 16). The expansion of the hypertrophic region in *Col2-Cre:Dicer^{fl/fl}* mice was not reversed in compound mutant mice, *Col2-caPPR:Col2-Cre:Dicer^{fl/fl}* mice, suggesting that the acceleration of hypertrophic differentiation in *Col2-Cre:Dicer^{fl/fl}* chondrocytes was caused by a defect either

Fig. 3. Reduced number of proliferating chondrocytes in the basisphenoidal-basioccipital growth plate. (A) Hematoxylin/eosin (H/E) staining and *Col10a1* expression in the basisphenoidal/basioccipital growth plate at E18.5 and P3.5. *Col2-Cre:Dicer^{fl/fl}* mice (cKO) show a progressive reduction in the width of the growth plate and the proliferating chondrocytes flanked by *Col10a1*-positive hypertrophic chondrocytes. Asterisks indicate the pituitary gland. (B) Reduced BrdU-positive chondrocytes in the cKO growth plate at P3.5.

independent or downstream of PTHrP receptor signaling (SI Fig. 7A). Because *Dicer* deficiency would affect multiple pathways, it is also possible that *Dicer* deficiency indeed affected PTHrP signaling, but because defects in other pathway played a dominant role, *caPPR* overexpression could not rescue the phenotype.

Dicer Is Required for Maintenance of Proliferating Chondrocytes in the Growth Plate. The skeletal growth defect and reduced width of the growth plate suggested a decrease in number of chondrocytes in *Col2-Cre:Dicer^{fl/fl}* mice. Indeed, the reduction in proliferating chondrocytes in *Col2-Cre:Dicer^{fl/fl}* mice was particularly well demonstrated in the growth plate between the basisphenoidal and basioccipital bones in the skull base (Fig. 3). This bidirectional growth plate had two *Col10a1* domains flanking the region containing proliferating chondrocytes. The basisphenoidal-basioccipital growth plate of *Col2-Cre:Dicer^{fl/fl}* mice was thinner in width, and the domain of proliferating chondrocytes progressively became smaller (Fig. 3A). The diminished number of proliferating chondrocytes at P3.5 in *Col2-Cre:Dicer^{fl/fl}* mice was shown by the fused *Col10a1* domains and the reduction in the BrdU-positive domain (Fig. 3). Overexpression of *caPPR* failed to restore the mass of proliferating chondrocytes, again supporting that defective PTHrP receptor signaling is not the major cause for the phenotype (SI Fig. 7B). Loss of *Dicer* has been

reported to increase cell death in multiple types of cells (9, 17–21). We did not find an overt increase in cell death assessed by the TUNEL assay (SI Fig. 8C), and therefore it was unlikely that cell death contributed to the decrease in proliferating chondrocytes. In addition, we found a dramatic decrease in chondrocyte proliferation in *Col2-Cre:Dicer^{fl/fl}* mice (Fig. 4). These findings suggest that loss of *Dicer* results in a reduction in the number of proliferating chondrocytes through two distinct mechanisms: acceleration of differentiation into postmitotic hypertrophic chondrocytes and reduction in chondrocyte proliferation.

miRNA and Gene Expression Profiling Suggests Limited Direct Regulation of mRNA Expression by Chondrocytic miRNAs. Although *Dicer* has been shown to process RNA species besides premiRNAs in mammalian cells (22), it is believed that the primary role of *Dicer* in mice is to generate mature miRNAs. Therefore, we hypothesized that the reduction in levels of chondrocytic miRNAs caused the physiologic changes in *Dicer*-deficient chondrocytes. To understand the roles of miRNAs in chondrocytes, first we performed miRNA profiling using a bead-based array (23). miRNA profiles from different chondrocyte sources showed modestly different patterns, whereas the miRNA profile of calvarial cells showed greater dissimilarity from those of chondrocytes (SI Fig. 9 and SI Table 1). The majority of miRNAs

Fig. 4. Reduced chondrocyte proliferation in *Col2-Cre:Dicer^{fl/fl}* mice (cKO). The BrdU-labeling assay demonstrates a dramatic reduction in chondrocyte proliferation in cKO growth plates. The BrdU-labeling index was calculated in the periarticular (PA) and columnar proliferating (C) region of the proximal growth plate of the tibia at the indicated ages. Representative BrdU-staining pictures of P0.5 tibiae are shown. The BrdU-labeling index in cKO mice was significantly reduced in the columnar region at E16.5, and in both periarticular and columnar regions at P0.5. Asterisks indicate statistical significance with the $P < 0.05$.

Fig. 5. miRNA expression in chondrocytes. (A) Relative expression levels of the 30 most-abundant miRNAs in neonatal hindlimb cartilage (chondrocytes) and calvarial cells (osteoblasts). Many abundant miRNAs are similarly expressed in chondrocytes and osteoblasts. (B) Thirty miRNAs preferentially expressed in chondrocytes. The expression of muscle-specific miRNAs, miR-206, -133, and -1 could be overestimated because of possible contamination of muscle tissues to cartilage specimens. (C) Expression of 30 miRNAs in control and *Dicer*-deficient chondrocytes. RNA isolated from neonatal hindlimb cartilage was subjected to miRNA profiling. Expression levels of most of miRNAs were reduced by 60–70% in *Dicer*-deficient chondrocytes. Complete data are shown in [SI Table 2](#).

abundantly expressed in hindlimb chondrocytes and calvarial osteoblasts were evolutionary conserved and relatively ubiquitous ones such as members of the let-7 family, miR-16, and miR-26 (Fig. 5A). The “cartilage-specific” miRNAs, miR-140 and miR-140*, are expressed at high levels, a result consistent with previous reports (24, 25) (Fig. 5A). We also found that several miRNAs were preferentially expressed in chondrocytes compared with calvarial cells (Fig. 5B and [SI Fig. 9B](#)). miRNA profiling comparing control and *Col2-Cre:Dicer^{f/f}* mice showed that most miRNAs were reduced by ≈ 60 –70% in neonatal *Col2-Cre:Dicer^{f/f}* hindlimb growth plates (Fig. 5C and [SI Table 2](#)). We noticed that some miRNAs, such as miR-133 and miR-1, showed no reduction. This is probably due to contaminating muscle tissues into microdissected cartilage specimens, because these miRNAs are expressed at very high levels in muscle (26, 27). The observation that certain amounts of miRNAs were still detectable in the *Col2-Cre:Dicer^{f/f}* sample was unexpected. Considering the relatively proportional reduction in the majority of miRNAs and the efficient elimination of *Dicer* mRNA, it is unlikely that contamination of noncartilaginous tissues or inefficient *Dicer* deletion is the reason for the remaining miRNAs. Because mature miRNAs appear quite stable (28), these miRNAs in mutant growth plates are probably a carryover of preexisting miRNAs.

Next, we performed microarray analysis to identify genes

whose expression was altered in *Dicer*-deficient chondrocytes. RNA isolated from whole growth plates of P3.5 control and *Col2-Cre:Dicer^{f/f}* hindlimbs was subjected to microarray analysis. Of the 45,036 probes, 2,658 showed >1.5 -fold expression changes ([SI Table 3](#)). To test whether the reduction of miRNAs directly influenced RNA levels of miRNA-target genes in *Dicer*-deficient chondrocytes, we examined expression levels of predicted miRNA-target genes that had binding sites for five or more different seed sequences of the 30 most-abundant chondrocytic miRNAs using the microarray data. These miRNAs had 17 different seed sequences (seven-nucleotide sequences in positions two to eight of mature miRNAs) that were used to predict target genes by the computer program, TargetScanS (29). A total 4,310 genes were predicted as potential target genes of these miRNAs abundantly expressed in chondrocytes ([SI Table 4](#)). Two hundred and fourteen genes (5.0%) had binding sites for five or more different seed sequences. Among these genes, analysis of 71 genes whose expression data were available showed that most were either not expressed or had unchanged expression ([SI Fig. 10A](#)). Only *Hmga2* was found up-regulated in the *Col2-Cre:Dicer^{f/f}* growth plate, a result subsequently confirmed by quantitative RT-PCR and *in situ* hybridization ([SI Fig. 10B](#)). This finding is consistent with the reports that *Hmga2* transcripts were destabilized by miRNAs, let-7 and miR-98 (30–32). We also examined the 3'-UTR sequences of the 1,437

Primer sequences were: Dicer1-F, 5'-AATTGGCTTCCTCTGGTTAT-3' and Dicer1-R, GTCAGTCTCTCTCTCTCT-3'; β -actin-F, 5'-GCACTGTGTTGCATAGAGG-3' and β -actin-R, 5'-GTTCCGATGCCCTGAGGCTC-3'.

Microarray Analysis. RNA was isolated from microdissected whole growth-plate cartilage of the distal femur of neonatal mice by using the RNeasy mini Kit (Qiagen). Up to 10 limbs were pooled for a group. Two samples per each group were separately prepared. The biotinylated cRNA was hybridized to the Affymetrix MOE430 v2 chip. Normalization and comparison between groups were performed by using the dChip (53). Genes considered to be

absent in more than two sample sets based on the Affymetrix detection (present/absent) call were removed from the comparison analysis.

ACKNOWLEDGMENTS. We thank Greg Nachtrab for technical assistance, Dr. Changzhong Chen and Dr. Edward Fox at the Dana Farber Cancer Institute for help in Affymetrix microarray analysis, and Dr. Vamsi Mootha and David Keller at the Broad Institute for MotifADE analysis. T.K. is supported by National Institutes of Health (NIH) Grant 1R21AR054500. Part of this work is supported by NIH Grant DK56246 (to A.P.M. and H.M.K.). S.J.R. was supported by post-doctoral fellowships from the National Health and Medical Research Council of Australia (Grant 301299) and the Arthritis Foundation (Grant 401683).

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