The RNaseIII enzyme *Dicer* is required for morphogenesis but not patterning of the vertebrate limb

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The RNaseIII-containing enzyme *Dicer* is believed to be required for the processing of most, if not all, microRNAs (miRNAs) and for processing long dsRNA into small interfering RNAs. Because the complete loss of *Dicer* in both zebrafish and mice results in early embryonic lethality, it has been impossible to determine what role, if any, *Dicer* has in patterning later tissues in the developing vertebrate embryo. To bypass the early requirement of *Dicer* in development, we have created a conditional allele of this gene in mice. Using transgenes to drive Cre expression in discrete regions of the limb mesoderm, we find that removal of *Dicer* results in the loss of processed miRNAs. Phenotypically, developmental delays, in part due to massive cell death as well as disregulation of specific gene expression, lead to the formation of a much smaller limb. Thus, *Dicer* is required for the formation of normal mouse limbs. Strikingly, however, we did not detect defects in basic patterning or in tissue-specific differentiation of *Dicer*-deficient limb buds.

microRNAs | mouse

MicroRNAs (miRNAs) are small, noncoding RNAs, ≈22 nt long. Large numbers of miRNAs are encoded in the genomes of all metazoans, including, at minimum, several hundred embedded in the genome of higher vertebrates such as mice and humans (1, 2). Animal miRNAs were first identified in *Caenorhabditis elegans* by mutations causing strong developmental phenotypes (3–5). Genetic and biochemical studies revealed that miRNAs act by mediating cleavage and/or inhibiting translation of specific target genes (6). These genes are targeted by virtue of regions complementary to a core sequence in the respective miRNAs. On this basis, several thousand putative targets have now been predicted for vertebrate miRNAs, and several intriguing targets have been validated *in vivo* and *in vitro* (1, 2, 7). These data, together with functional data emerging from studies in *C. elegans* and *Drosophila* (3, 8–10), support a growing belief that miRNAs may play critical roles in controlling key developmental events in vertebrates as well as invertebrates. However, direct demonstrations of the necessity of specific miRNAs for vertebrate development has been slow in coming, largely because vertebrate miRNAs exist as families of highly related or even identical sequences (11). This high level of possible redundancy suggests that simple loss-of-function approaches will be less than fruitful for studying miRNA function in vertebrate development.

An alternative, albeit heavy-handed, approach to testing the necessity of miRNAs in higher animal development is to create mutations in the upstream enzymes responsible for processing miRNAs to their mature, active form. Mature animal miRNAs are ≈22 nt in length, generated by sequential processing by a series of RNaseIII-related enzymes. *Drosha* produces a primary miRNA hairpin transcript of ≈70 nt from a longer precursor RNA (12). This ≈70-nt hairpin is then cleaved by a second RNaseIII enzyme, *Dicer*, to yield the 22-nt mature miRNA (13). Importantly, there is only a single copy of *Dicer* in the mouse genome. Thus, targeted deletion of *Dicer* should, in principle, yield mice deficient of all mature miRNAs. *Dicer* is also required for processing dsRNA transcripts into small interfering RNAs; hence, small interfering RNAs as well as the ability to respond to exogenously provided dsRNA would also be expected to be compromised in a *Dicer* mutant.

In vertebrates, *Dicer*-null mutations have been created in both mice and zebrafish (14–16). Mice that lack *Dicer* survive until embryonic day (E) 7.5, possibly because of the presence of maternal *Dicer* protein (15). These embryos die before axis formation, consistent with the model that *Dicer* products might be crucial for embryonic patterning and morphogenesis. In zebrafish, removal of zygotic *Dicer* does not result in loss of miRNA-processing activity in early embryogenesis (16), presumably because of the presence of maternal *Dicer*. These embryos have no obvious defects in their early development, although they exhibit a developmental delay before finally dying at 14–21 days after fertilization. Zebrafish mutant embryos lacking both maternal and zygotic *Dicer* protein show greater effects. Surprisingly, however, these embryos still survive through early embryogenesis and appear to correctly pattern and correctly specify many early cell types (14). As embryogenesis proceeds they undergo abnormal morphogenesis of the brain, heart, and other organs. Although the examination of early embryos formed in the absence of *Dicer* activity has not revealed any defects in cell fate specification, *Dicer*-deficient embryonic stem cells are defective in differentiation both *in vitro* and *in vivo*, suggesting that some aspects of differentiation are under the control of *Dicer*-dependent factors (17).

The inability of *Dicer*-null embryos to survive until later stages of development and, in particular, of *Dicer*-deficient mice to survive past E7.5 has inhibited study of the later functions of this unique enzyme, including the role mature miRNA may have in later development. Therefore, it is still uncertain how widespread is the requirement for *Dicer* and miRNAs in vertebrate development. To overcome the early lethality associated with the null mouse allele, we have created a cre-inducible conditional allele of *Dicer*. This conditional allele can be used to remove *Dicer* function in any tissue in which the site-specific cre recombinase can be expressed. Using the *Dicer* conditional allele and transgenic mouse lines that express cre in the limb mesoderm, we provide an initial characterization of the role that *Dicer* plays in vertebrate limb development.
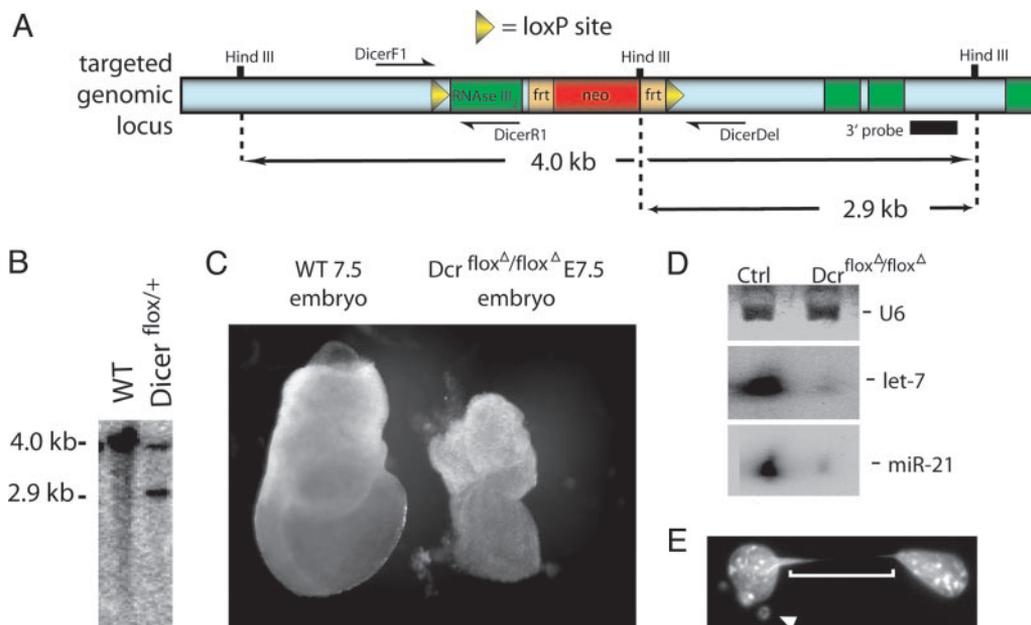
Materials and Methods

Creation of a *Dicer*-Null Conditional Allele. The *Dicer* conditional allele (*Dicer^flox^*) was created by inserting loxP sites around an

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Fig. 1. Construction of a *Dicer* conditional mouse allele. (*A*) Schematic of the *Dicer* conditional targeting construct. (*B*) Southern blot of correctly targeted embryonic stem cells. By using a 3′ probe, a 2.9-kb fragment is detected in addition to the wild-type 4.0-kb fragment in targeted embryonic stem cells. (*C*) Upon Cre-mediated recombination of the *Dicer^flox^* allele in the germ line, homozygous mutant embryos resemble the reported null allele (15). (*D*) RNA was extracted from primary fibroblasts from wild-type or *Dicer^flox^*/*Dicer^flox^* mice. Parallel experiments with GFP adenovirus indicated near 90–100% infection efficiency. The presence of a small amount of processed *let-7* and *miR-21* RNA in *Dicer*-null cells may be due to the inclusion of *Dicer*-positive cells in our RNA preparations or may indicate that a small amount of miRNA processing is independent of *Dicer*. (*E*) A representative example of a cell displaying defects in chromosome segregation upon removal of *Dicer*. The arrowhead points to a micro nucleus, and the brackets indicate an anaphase spindle. Counting the number of anaphase spindles and satellite nuclei present among four randomly chosen fields of DAPI-stained cells revealed that ≈25% of the *Dicer*-null cells displayed defects in chromosome segregation and accumulation of satellite nuclei despite a normal morphological appearance under the light microscope. In control adeno-GFP-treated cells, 1.4% of the cells contained satellite nuclei but no anaphase bridges.

exon that encodes most of the second RNaseIII domain (Fig. 1). Cre-mediated recombination resulted in the removal of 90 aa. Upon cre-mediated recombination, an antibody made against a region of the *Dicer* protein 3′ to the deletion was still able to recognize *Dicer* protein, suggesting that removal of this exon does not result in a downstream frame-shift or an unstable protein (data not shown). *Dicer* was conditionally removed from the limb mesenchyme by using the previously described Cre lines *prx1cre* (18) and *Shhgfpcre* (19). The conditional floxed allele was genotyped by using primers DicerF1 (CCTGACAGTGACG-GTCCAAAG) and DicerR1 (CATGACTCTTCAACT-CAAACT). This PCR combination flanks the 5′ loxP site. The floxed allele produced a 420-bp PCR product whereas a wild-type allele resulted in a 351-bp product. The deletion allele was genotyped by using primers DicerF1 and DicerDel (CCTGAG-CAAGGCAAGTCATTC). The DicerDel primer is downstream of the neomycin cassette. The deletion allele produced a 471-bp PCR product whereas a wild-type allele resulted in a 1,300-bp product. Mice homozygous for the conditional allele or containing a conditional allele and a recombined deletion allele were indistinguishable from wild-type littermates. The neomycin cassette has not been removed in the floxed *Dicer* allele, suggesting that it does not interfere with wild-type *Dicer* expression.

Small RNA Northern Blots, Immunohistochemistry, and *in Vitro* Cell Culture. Small RNA Northern blots were performed as in ref. 35. Derivation, culture, and cre infection of mouse embryonic fibroblasts follow standard protocols. Phosphorylated extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase antibody staining was performed as described in ref. 20. Skeletal preparations were performed as in ref. 21, and whole-mount *in situ* hybridizations using digoxigenin-labeled RNA probes were performed as described in refs. 22 and 23. β-Ga-

lactosidase staining was performed as in ref. 19. To examine cell death in embryos in which *Dicer* had been removed from the limb mesoderm (*Dicer^flox^*/*Dicer^flox^*;*prx1cre*), we used acridine orange (24). A working stock of 5 mg/ml acridine orange was diluted 1:10,000 in PBS. Dissected embryos were transferred to the working solution of acridine orange and incubated for 30 min at 37°C in the dark. Embryos were then washed twice in PBS for 5 min and viewed with a fluorescence microscope.

Results

Recombination of the *Dicer* Conditional Allele Results in Loss of miRNA Processing. Because *Dicer*-null mouse embryos arrest development at E7.5 (15), well before the limbs and most other organ systems form, we constructed a conditional allele of this gene by flanking an exon encoding most of the second RNaseIII domain with *loxP* sites (see *Materials and Methods* and Fig. 1). This deletion would be predicted to abolish the enzymatic activity of *Dicer*. To confirm that the removal of the *Dicer* floxed exon produced a null allele, we removed this exon in the germ line by crossing the floxed allele to a germ-line cre (β-actin cre). Homozygous *Dicer^floxΔ^*/*Dicer^floxΔ^* embryos arrested at E7.5 and were similar in appearance to the reported *Dicer*-null mutant (Fig. 1*C*) (15).

Because *Dicer* has been postulated to process miRNAs, we investigated *in vitro* whether mature miRNAs were produced in cells that lacked the *Dicer* conditional allele. Primary fibroblasts were prepared from embryos homozygous for the floxed *Dicer* allele. Cre was introduced into these cells by using an adenovirus vector (a gift from Beverly Davidson, University of Iowa, Iowa City). Using PCR, we detected no wild-type alleles of *Dicer* after adenovirus infection (data not shown), suggesting that the infection of the cell culture by the adenovirus was complete. To determine whether miRNAs were processed in Dicer-minus
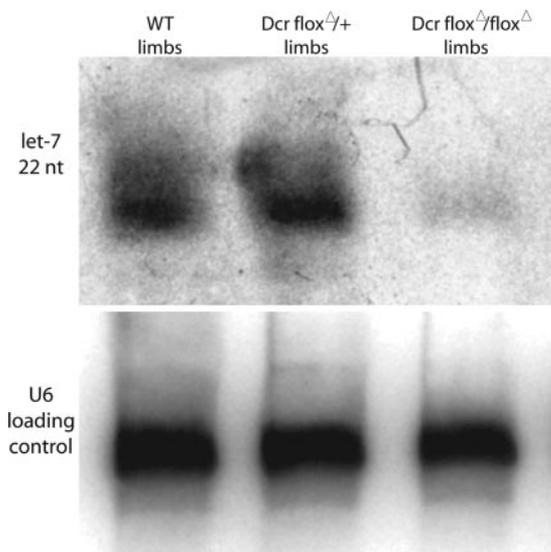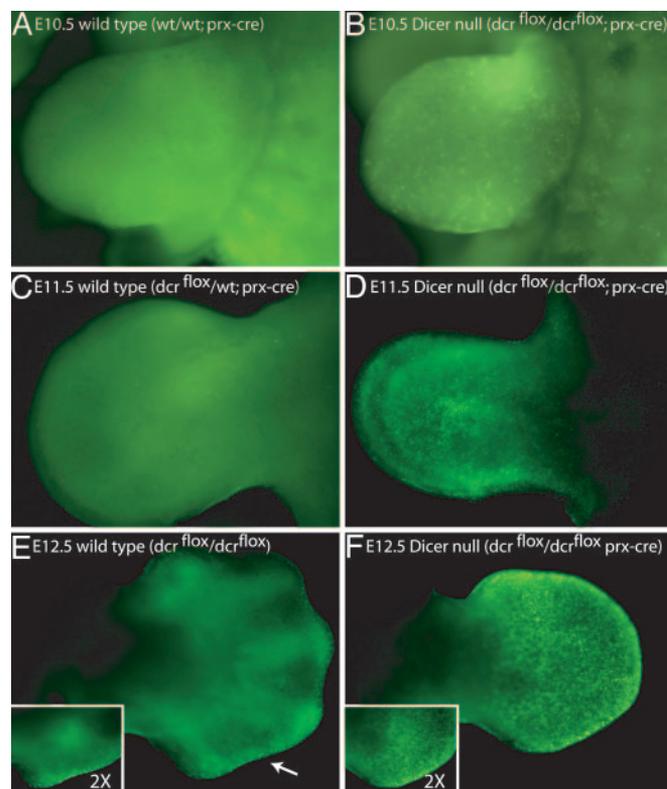
Fig. 2. *let-7* is not correctly processed in limbs that lack *Dicer*. RNA was extracted from wild-type, heterozygous, or *Dicer*-null E12.5 forelimbs. The small amount of mature *let-7* present in *Dicer^flox/Dicer^flox;prx1cre* forelimbs may result from the correct processing of *let-7* in the limb ectoderm [*let-7* is known to be expressed in the limb ectoderm (35), and the *prx1cre* allele removes *Dicer* activity only from the limb mesoderm].

Fig. 3. Loss of Dicer in the mouse limb leads to cell death. *A*, *C*, and *E* show wild-type limbs, and *B*, *D*, and *F* show limbs in which Dicer has been removed from the limb mesoderm using the *prx1cre* allele. An increase in cell death in *Dicer*-minus limbs compared with age-matched littermates was observed in E10.5 limbs (compare *A* and *B*). By E11.5, Dicer-minus limbs were narrower than wild-type limbs and exhibited an increase in cell death throughout the limb mesoderm. Note that loss of a single copy of *Dicer* does not lead to an increase in cell death (compare *C* and *D*). By E12.5, a wild-type pattern of cell death was observed in the apical ectodermal ridge (AER) of wild-type limbs (arrow in *E*). Dicer-minus limbs exhibited cell death in the AER in addition to ectopic cell death throughout the limb mesoderm (*F*). Dicer-minus limbs were also smaller and digit condensations were not visible in E12.5 mutant limbs. The ×2 magnification insets in *E* and *F* are of the distal, posterior region of the respective limbs. All pictures are of forelimbs from littermates. Images in *A*–*D* were taken at the same magnification.

fibroblasts, we examined the processing of two miRNAs, *let-7* and *miR-21*. Whereas both of these miRNAs were easily seen as 22-nt processed forms in control primary cells, the cultures infected with the Cre adenovirus showed barely detectable levels of processed *let-7* and *miR-21* (Fig. 1*D*). The remaining processed miRNAs can be attributable to perdurance of *Dicer* protein or processed miRNAs after genomic recombination of the *Dicer* locus or may suggest that low levels of miRNA processing can occur in the absence of *Dicer*. After removal of *Dicer* activity in these primary fibroblasts, no overt differences in cell morphology were observed, although the cells exhibited a reduction in growth rate. By the third passage, dividing cells could be observed to contain nuclear bridges and microsatellites in up to 25% of the cells in the culture (Fig. 1*E*), consistent with previous reports of the effect of loss of *Dicer* after extended cell passage (24–26).

Dicer Is Required for Regulating Limb Size. To investigate the *in vivo* role of *Dicer* in limb development, we removed this gene specifically from the entire limb mesoderm using the *prx1cre* transgene (18). The *prx1cre* allele is a transgene that expresses Cre throughout the limb mesoderm in both the mouse forelimb and hindlimb. In the forelimb of *prx1cre* mice, Cre expression is observed in the limb mesoderm beginning at E9.5, whereas expression in the hindlimb commences ≈1 day later (18). No Cre expression is observed in the limb ectoderm using this allele.

Upon *prx1cre*-dependent removal of *Dicer* in the entire limb mesoderm, consistent with our *in vitro* data, the vast majority of miRNAs were not processed (Fig. 2). Moreover, morphologically, we observed a striking decrease in limb size. In the forelimb, the limb was visually indistinguishable from those of age-matched littermates from E9.5 (when a limb bud forms) until E11. At E11, *Dicer*-minus forelimbs were smaller than those of age-matched littermates, and by E12.5 mutant limbs resembled the size of E11.5 limbs. Limbs that lacked *Dicer* remained smaller than age-matched controls from E11 onward. This phenotype is also observed in the hindlimb but is not as severe, possibly because of the relative delay of *prx1cre* expression in the hindlimb. Consistent with these results, a reduction in the size of

multiple organs upon removal of *Dicer* has also been reported in zebrafish (16).

Loss of Dicer Results in Ectopic Cell Death but Not Defects in Pattering of Differentiation of the Vertebrate Limb. To understand the decrease in size of the *Dicer*-mutant limb buds in *Dicer^flox*/*Dicer^flox;prx1cre* embryos, we examined various cellular parameters. No overt change in cell proliferation was seen *in vitro* after removal of *Dicer* activity (data not shown). We therefore turned our attention to the possibility that the decrease in limb size was due to an increase in programmed cell death. We stained the limbs of live embryos with acridine orange, a marker of cells undergoing apoptosis (27). In the forelimbs of E10 *Dicer*-minus embryos, no increase in cell death was observed (data not shown). Similarly, in the hindlimb, no ectopic cell death in E10, E10.5, or E11 limbs was found. However, starting at E10.5 in the forelimbs and E12.5 in the hindlimbs, significant cell death was observed throughout the limb mesoderm in limbs that lacked *Dicer* (Fig. 3).

Strikingly, however, when postnatal limbs were examined, all of the differentiated cell types normally found in the mature limb bud appeared to be present in the *Dicer*-deficient limbs. More-
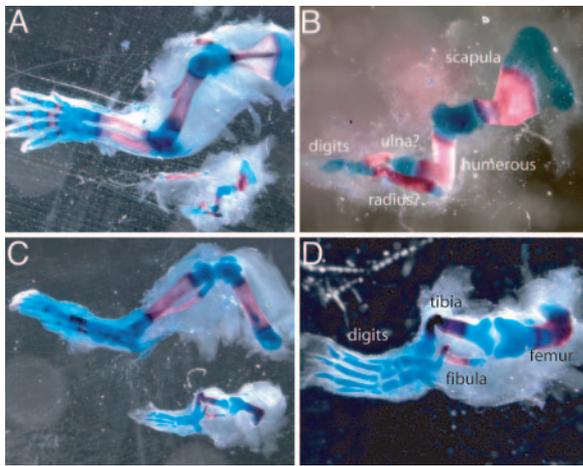
Fig. 4. Skeletal preparations of E20 *Dicer*-minus limbs. (*A*) The upper image shows a wild-type E20 forelimb, and the lower image shows a *Dicer^flox^/Dicer^flox^;prx1cre* forelimb from an age-matched littermate. (*B*) Magnification of the mutant limb shown in *A*. Notice that despite its small size the mutant limb contains most of the bones associated with normal limb development. (*C*) The upper image shows a wild-type E20 hindlimb, and the lower image shows a *Dicer^flox^/Dicer^flox^;prx1cre* hindlimb from an age-matched littermate. (*D*) Magnification of the mutant limb shown in *C*.

over, although morphological malformations were apparent, these did not appear to be due to patterning defects. The long bones of the arm and leg were twisted in appearance, and development from cartilage into bone was delayed, both features consistent with the decrease in size of the developing limb bud (28, 29). Importantly, however, analysis of skeletal preparations of *Dicer*-minus mouse forelimbs and hindlimbs revealed that all proximodistal segments were present (Fig. 4). The *Dicer*-minus forelimbs did show a notable reduction in the number of digits as well as some digit fusions. These could have resulted from the decrease in the width of the handplate during limb development but alternatively could have represented a forelimb-patterning defect. To distinguish between these possibilities, we used an *Shhgfpcre* allele to remove *Dicer* at E9.75, at the onset of *Shh* expression, specifically from the cells of the zone of polarizing activity (ZPA), a known signaling center responsible for patterning the anterior/posterior limb axis. During normal limb development, cells that have at one time expressed *Shh* (cells of the ZPA) form digits 5 and 4 and part of digit 3 (19). An increase in apoptosis of cells that lacked *Dicer* was observed in a subset of cells expressing the *Shhgfpcre* allele, consistent with our observation of an increase in cell death throughout the entire limb mesoderm after global removal of *Dicer* (data not shown). Importantly, limbs in which *Dicer* was removed from the ZPA did not contain defects in anterior/posterior patterning, and *Dicer*-minus cells that did not die were still found in the distal/posterior region of the autopod and formed digits 5 and 4 and part of digit 3 of both the forelimbs and hindlimbs (Fig. 5 and data not shown) demonstrating that, at least for cells that have at one time expressed *Shh* in the limb, *Dicer* is not required for their patterning.

Developmental Delay in the Expression of a Subset of Genes in Dicer-Deficient Vertebrate Limbs. Consistent with the lack of morphological patterning defects, we saw no alteration in the spatial expression patterns of genes known to play critical roles in limb patterning, including *Shh*, *Fgf8*, *Bmp-4*, *Bmp-7*, *MyoD*, and *Gdf5*, although at later stages these displayed a developmental delay such that they were expressed in patterns that were consistent with an earlier stage of limb development compared with age-matched
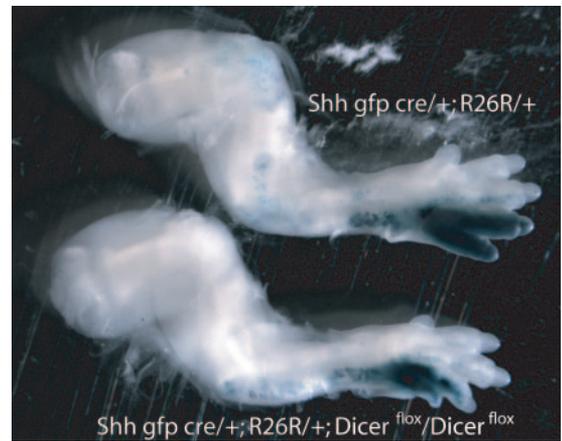

Fig. 5. Loss of *Dicer* in cells that express *Shh* does not alter *Shh* signaling or cell migration. Removal of *Dicer* in *Shh* cells of E18.5 embryos was accomplished by mating *Dicer^flox^/+;Shhgfpcre/+* males with *Dicer^flox^/Dicer^flox^;R26R/+* females. Using the R26R allele, we were able to identify and follow the fate of cells that expressed the *Shhgfpcre* allele, had undergone a recombination event activating the *LacZ* gene, and lacked *Dicer*. Previously, we had demonstrated that cells that express *Shh* in the ZPA migrate to the distal limb and form digits 5 and 4 and part of digit 3 (19). In limbs in which *Dicer* was removed in *Shh*-expressing cells, abnormal cell migrations were not observed. No blue cells (*Dicer*-minus) were observed in abnormal locations compared with wild-type age-matched littermates.

littermates (Fig. 6 and data not shown). One gene family displaying this property was potentially of particular relevance to the morphological phenotype. In wild-type limbs, *Spry* genes, a family of receptor tyrosine kinase (RTK) signaling antagonists, are initially expressed broadly but then become restricted in their expression to the distal mesenchyme during midlimb bud stages, reflecting their normal dependence on *Fgf* signaling (30). In contrast, in age-matched *Dicer^flox^/Dicer^flox^;prx1cre* littermates we observed a significant increase in *Spry2* expression throughout the midlimb bud mesenchyme (compare Fig. 6 *E* and *F*). Intriguingly, both the decrease in size of the early limb bud and the subsequent normally patterned limb skeleton with reduced and twisted skeletal elements observed in *Dicer*-mutant limbs are very reminiscent of the phenotype obtained upon viral overexpression of *Spry* genes in the chick limb (30).

If the increase we observe in *Spry* gene expression contributes to the cell death observed at E10.5 in the forelimb of conditional *Dicer* mutants, this overexpression should be reflected in a decrease in *FgfR* and other RTK signaling in the limb bud at E10.5 when cell death is first observed. To test this hypothesis, we made use of an antibody specific for the phosphorylated form of ERK, a downstream component of RTK signaling (31). In immunohistochemistry on sectioned E10.5 wild-type forelimb buds, there is a high level of phospho-ERK-specific staining in the distal limb directly under the ectoderm and a clearly evident graded staining at a lower level throughout the rest of the mesenchyme (Fig. 7*A*). In contrast, parallel sections of *Dicer*-deficient limb buds show the same level of intense staining at the distal margin but a complete loss of detectable phospho-ERK in the rest of the mesenchyme, correlating with elevated levels of *Spry* gene products throughout the mesenchyme (Fig. 7). These results suggest that the elevation of *Spry* genes in the developmentally delayed *Dicer*-deficient limbs results in decreased RTK signaling. These observations are consistent with the observed increase in mesenchymal cell death upon removal of *Dicer* (see Fig. 3).

Discussion

Constructing a unique mouse conditional allele of *Dicer* has allowed us to examine the role played by *Dicer* in vertebrate limb develop-

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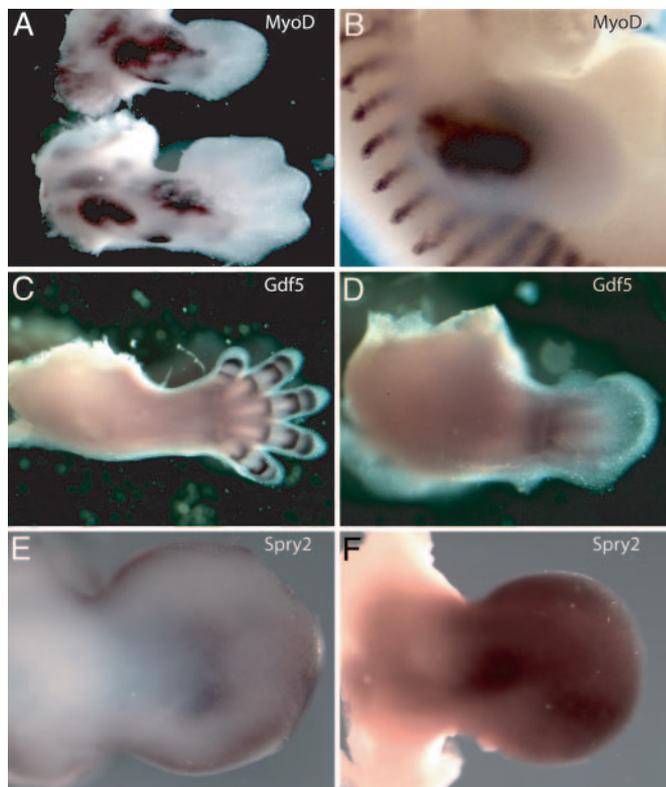
Fig. 6. Gene expression in a *Dicer*-minus limb. (*A*) RNA *in situ* hybridizations for *MyoD* (muscle) staining. The upper image shows a *Dicer^flox^/Dicer^flox^;prx1cre* E12.5 forelimb, and the lower image shows a *Dicer^flox^/+;prx1cre* control limb. (*B*) RNA *in situ* hybridizations for *MyoD* staining in an E11.5 wild-type forelimb. Notice that the staining pattern in an E12.5 *Dicer^flox^/Dicer^flox^;prx1cre* E12.5 forelimb is similar to *MyoD* expression in an E11.5 limb. (*C*) RNA *in situ* hybridizations for *Gdf5*, a marker of joint formation. Staining is in an E13.0 wild-type forelimb. (*D*) *Gdf5* staining in a *Dicer^flox^/Dicer^flox^;prx1cre* forelimb (×2 magnified compared with *C*). All control limbs are from age-matched littermates. (*E* and *F*) *Spry2* whole-mount RNA *in situ* hybridizations on *Dicer^flox^/Dicer^flox^* (*E*) and *Dicer^flox^/Dicer^flox^;prx1cre* (*F*) E11.75 littermates, respectively. Notice that *Spry2* is expressed at much higher levels in mesoderm that lacks *Dicer* (*F*). For all probes, more than four mutant and wild-type embryos were examined.

ment. We demonstrate that *Dicer* is required for proper morphogenesis of the vertebrate limb, although the nature of the defects we observe are not as severe as might have been expected.

All of the morphological defects we observe can be explained on the basis of a global decrease in the number of cells in the developing limb bud. Limb buds of *Dicer*-deficient limbs were notably smaller in size from E11 onward. The skeletal elements that formed in these limbs were smaller than normal, were developmentally delayed, and formed a reduced number of digits in a narrower-than-normal hand plate. The reduction in number of digits could, in principle, also be explained by a decrease in signaling from the ZPA or other disruptions in anterior/posterior patterning. However, early patterning gene expression is normal in these limbs. Moreover, removal of *Dicer* activity specifically in the ZPA does not affect patterning of posterior limb structures, arguing that the digit reductions are indeed related to the decrease in size of the limb bud and not a patterning defect. In addition, the skeletal elements that form in *Dicer*-deficient limbs displayed fusions and were strikingly twisted and bent. The twisted bone phenotype is most likely not due to defects in patterning the early limb, because the twisting occurs after condensation of the primordia of the skeletal elements. This class of skeletal defect has been previously seen
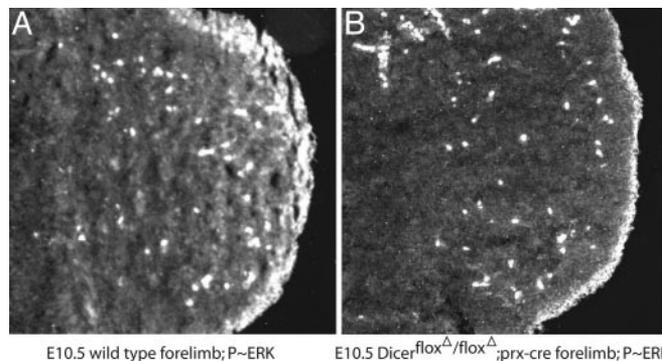
Fig. 7. FGF signaling is reduced in limb buds lacking *Dicer* activity. Frontal sections through E10.5 control (*A*) and *Dicer^flox^/Dicer^flox^;prx1cre* (*B*) limbs were immunostained with an antibody against phosphorylated ERK/mitogen-activated protein kinase (MAPK), an indicator of active FGF and other RTK signal transduction as described in ref. 20. Phosphorylated ERK/MAPK is detectable at high levels in the mesoderm directly apposed to the apical ectodermal ridge (AER), a potent source of FGF signals, and is present in a gradient extending proximally through the mesoderm from the distal tip (*A*) (31). In contrast, in limb buds lacking *Dicer* activity (*B*), this graded signal is absent, indicating a reduction in FGF/RTK signaling. High levels of phosphorylated ERK/MAPK are still detected beneath the AER, consistent with maintained FGF signaling in the limb ectoderm.

in limb buds displaying a reduction in size, e.g., due to *Goosecoid* misexpression in chicks (28) and in compound mutations in *Prx1* and *Prx2* mice (29).This phenotype can, in fact, be mimicked by the blocking of cell proliferation in chick limb buds (32), indicating that a decrease in the size of the limb primordia is sufficient to lead to the twisting of the skeletal elements.

The cause of the decrease in size of the limb bud, and consequent developmental delay and morphological defects, appears to be an increase in apoptosis throughout the limb mesenchyme. We observed no overt change in cell proliferation after *Dicer* removal. In contrast, a cell death study revealed a dramatic increase in apoptosis starting at E10.5 in the forelimb.

The increased cell death observed in limbs lacking *Dicer* activity is potentially due to a requirement for the enzyme at two levels: for specific modulation of *Spry* gene expression and possibly for assuring proper chromatin segregation, although our experiments do not address the extent to which the cell death observed upon loss of *Dicer* is due to one or the other of these factors. The chromatin segregation defects we observed *in vitro* are consistent with prior studies of cells deficient in *Dicer* activity in fission yeast (26, 33, 34). It is extremely likely that in the absence of *Dicer* activity similar defects occur *in vivo* as well. It is also likely that, as in cell culture, these defects manifest only after the *Dicer* products critical for this process have been diluted through multiple cell divisions or degraded sufficiently. Based on our *in vitro* work, such defects in chromatin specification would not be expected for six to eight cell divisions. In contrast, we observed an increase in cell death in the forelimb bud, with a time frame allowing for only three to four cell divisions after expression of the Cre transgene. This finding suggests that additional *Dicer*-regulated factors, not found in *Dicer*-minus cell lines studied to date, may be functioning in the limb mesenchyme.

Increased apoptosis in *Dicer*-minus limbs may, in part, result from the up-regulation of *sprouty* genes in *Dicer*-deficient limb buds. *Spry2* expression is significantly increased throughout the midlimb bud. Moreover, as an RTK antagonist, this increase is correlated with the expected decrease in detectable phospho-ERK throughout the limb bud. During limb development, RTK-mediated Fgf signaling is required in the distal limb to block apoptosis, and total loss of the source of Fgf protein in the distal ectoderm results in distal

cell death and skeletal truncation (31). More proximal regions of the limb mesenchyme do not depend on Fgf signaling but may require other factors signaling through RTK-mediated pathways for their survival. The observed increase in *Spry2* would result in a decrease in RTK signaling throughout the limb bud but not a complete loss of such signaling. Hence, the level of cell death is not high enough to cause truncation or amelia but rather results in a decrease in the amount of mesenchyme in the limb bud.

To test whether the observed increase in *spry* expression is, in itself, sufficient to induce the level of cell death we observed in *Dicer*-deficient limb buds, one would want to increase the level of *Spry* expression in the absence of *Dicer*-related changes, e.g., by viral misexpression in chick embryos. Such an experiment has been done previously (30). When *Spry2* is misexpressed throughout the limb mesenchyme, there is indeed a resultant decrease in the size of the early limb bud very similar to that observed in the mouse *Dicer* mutant (see Fig. 3 and ref. 30). Such limbs also develop shortened, twisted skeletal elements as observed in the absence of *Dicer*. Additionally, the limbs in which *Spry* has been misexpressed show a block or delay in ossification that we do not see in *Dicer*-deficient embryos. This difference can be explained by the continued high level of *Spry* activity present throughout late limb development in the case of viral misexpression whereas *Dicer*-deficient limb buds display elevated *Spry* expression only in the early limb mesenchyme. Nonetheless, at early bud stages when the expression of *Spry* is equivalent in the two cases, the viral misexpression experiment shows that high levels of *Spry* are sufficient to induce the pattern of limb defects one observes in *Dicer*-deficient *Spry* overexpressing mouse limb buds.

The *Spry* up-regulation we observed in *Dicer*-deficient limb buds could be a reflection of the general developmental delay in these limbs and hence an indirect consequence of the loss of *Dicer* activity. Indeed, in the earliest limb buds *Spry2* is expressed throughout the limb mesenchyme. However, it is also possible that the *Spry2* gene may be a direct target of miRNAs. In support of this hypothesis, a recent report (7) has identified eight miRNAs that have the potential to interact directly with the 3′ UTR of *Spry2*.

Using miRNA arrays we have confirmed that five of eight of these miRNAs are expressed in the vertebrate limb (unpublished results).

Dicer has been implicated in a number of processes during development, including processing of miRNAs, chromatin remodeling, and gene silencing. Our conditional allele removes the second *Dicer* RNaseIII domain but leaves the rest of the protein intact. This allele drastically reduces or possibly eliminates all miRNA processing in the limb mesoderm and is clearly important for cell division. In addition, Cre-mediated recombination of the conditional allele in the germ-line phenocopies the reported *Dicer*-null allele. However, we cannot rule out the possibility that the conditional allele is not null for all aspects of *Dicer* function in the vertebrate limb.

Surprisingly, given the large number of miRNAs identified in vertebrates and their known ability to modulate gene activity, we did not observe differentiation or patterning defects upon loss of *Dicer* activity. These data do not rule out early patterning roles for *Dicer*, because many critical aspects of limb patterning occur in the earliest stages of limb development when *Dicer* protein or processed miRNAs might still be present in the limb cells in our conditional mice. However, from mid-bud stage on we see a dramatic decrease in miRNA processing. Moreover, at later stages all *Dicer* activity should be absent. Thus, it is striking that later patterning events, such as segmentation of cartilage, and differentiation of the various lineages in the limb, such as the sequential cellular transitions of endochondrial ossification, proceed normally. These data suggest that *Dicer* (and, as a consequence, we conclude miRNAs) are not involved in the specification of individual tissue types in the vertebrate limb. These data are consistent with the recent observation that zebrafish (14) also specify individual tissues types in embryos lacking both maternal and zygotic *Dicer*.

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