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Gli2 and p53 Cooperate to Regulate IGFBP-3-Mediated Chondrocyte Apoptosis in the Progression from Benign to Malignant Cartilage Tumors

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SUMMARY

Clinical evidence suggests that benign cartilage lesions can progress to malignant chondrosarcoma, but the molecular events in this progression are unknown. Mice that develop benign cartilage lesions due to overexpression of Gli2 in chondrocytes developed lesions similar to chondrosarcomas when they were also deficient in p53. Gli2 overexpression and p53 deficiency had opposing effects on chondrocyte differentiation, but had additive effects negatively regulating apoptosis. Regulation of *Igfbp3* expression and insulin-like growth factor (IGF) signaling by Gli and p53 integrated their effect on apoptosis. Treatment of human chondrosarcomas or fetal mouse limb explants with IGFBP3 or by blocking IGF increased the apoptosis rate, and mice expressing Gli2 developed substantially fewer tumors when they were also deficient for *Igf2.* IGF signaling-meditated apoptosis regulates the progression to malignant chondrosarcoma.

INTRODUCTION

The progression of neoplasia from a benign lesion to malignancy is thought to require the progressive accumulation of mutations deregulating cell growth control, apoptosis, and DNA stability. Although this progression is identified in tumors of epithelial origin, it has not been demonstrated in mesenchymal tumors, in part because benign precursor lesions for most sarcomas are not clearly identified. This is not the case for enchondromas, which are benign cartilaginous tumors located in the metaphysis of bone. Enchondromas can occur as isolated or multiple lesions and can progress to malignant chondrosarcoma, an event that occurs at a higher rate in multifocal diseases. Although the factors implicated in the progression to chondrosarcomas are poorly understood, cytogenetic and mutational analysis of tumor suppressor genes identified mutations or cytogenetic anomalies in the p53 gene in roughly a third of chondrosarcomas (Benini et al., 2006; Oshiro et al., 1998), suggesting that tumor suppressor gene inactivation is important in the transformation of enchondromas to malignant sarcomas. p53 regulates cell cycle progression and apoptosis (Komarova et al., 1997), acting as a transcription factor to regulate the expression of genes such as *p21* and *GADD45* and interacting with proteins such as the proapoptotic member of the Bcl2 family, Bax (Kobayashi et al., 2002; Reinke and Lozano, 1997; Srinivasula et al., 1998).

Bone development depends on proper coordination of spatial and temporal control of cell function. The growth plate is an integral component of endochondral bone development and is also responsible for postnatal longitudinal growth. Growth plate chondrocytes undergo an orderly process of proliferation and

SIGNIFICANCE

Although molecular mechanisms responsible for the progression of benign to malignant tumors of epithelial origin have been identified, they have not been demonstrated in mesenchymal tumors. Here, we used a mouse model of enchondromatosis to show that p53 deficiency can cause chondrosarcomas to arise from benign lesions. An unexpected role for IGFBP3 in this progression was found. Human cartilage tumors have low levels of *IGFBP3* expression, compared to normal chondrocytes, with chondrosarcomas having lower levels than benign lesions, suggesting that *IGFBP3* level is a prognostic factor in cartilage tumors. Furthermore, IGFBP3 treatment or IGF signaling blockade increased chondrosarcoma apoptosis, suggesting a therapeutic approach to chondrosarcomas, a tumor for which there is no universally effective chemotherapy.

differentiation, ultimately resulting in the production of extracellular matrix components, such as type X collagen (ColX), and programmed cell death. Bone forms initially in the central part of the bone, adjacent to the metaphyseal growth plate. Secondary ossification sites arise at the epiphyseal ends of the bone and progress toward the center of the bone shaft, eventually leading to closure of the growth plate at maturity. This process is tightly regulated by a number of signaling pathways that maintain the normal rate of endochondral bone growth (Ballock and O'Keefe, 2003).

Because of their location adjacent to the growth plate, it has long been suspected that enchondromas arise from rests of growth plate chondrocytes that fail to undergo terminal differentiation. This notion is supported by the development of metaphyseal cartilage rests, similar in nature to enchondromas in humans, in transgenic mice expressing either a mutant PTHR1 or the hedgehog (Hh)-activated transcription factor Gli2 in chondrocytes (Hopyan et al., 2002). PTHrP and the Hh signaling pathways play important roles in regulating growth plate chondrocyte differentiation. Activation of the PTHrP pathway delays chondrocyte differentiation (Amizuka et al., 1994; Karaplis et al., 1994; Vortkamp et al., 1996), resulting in a downregulation of expression of the Hh ligand, Indian Hedgehog (Ihh), by hypertrophic chondrocytes (Lanske et al., 1996). Ihh regulates chondrocyte proliferation and differentiation though direct regulation of Gli transcription factors and by regulating PTHrP ligand expression (Kobayashi et al., 2002; Koziel et al., 2005; Mau et al., 2007). As such, these signaling pathways act in a feedback loop regulating differentiation. In the case of enchondromatosis, there is deregulation of this feedback loop, resulting in constitutive activation of Hh signaling, even in the presence of PTHrP stimulation and Gli-mediated transcriptional activation (Hopyan et al., 2002; Tiet et al., 2006).

Although malignant and benign cartilage lesions exhibit constitutive Gli-mediated transcription, the level of Gli transcriptional activation is similar for both tumor types, suggesting that while constitutive Hh signaling is a common finding in cartilage tumors, a higher level of activation is not responsible for malignant progression (Tiet et al., 2006). Other factors, such as inactivation of tumor suppressor genes, are thus likely important in cartilage tumor progression. As such, the aim of this study was to investigate the effect of p53 deficiency on the progression of enchondromas.

RESULTS

Deficiency of p53 in Gli2-Transgenic Mice Predisposes to the Development of Larger, More Cellular, Cartilage Lesions

We utilized a mouse model of enchondromatosis in which the Hh-activated transcription factor, *Gli2*, is expressed in chondrocytes driven by the regulatory elements of type two collagen, *Tg(Gli2;ColIIAI)* (Hopyan et al., 2002). The tumors in these mice develop on the basis of a molecular feature that is common in enchondromas—constitutive activation of Gli-mediated transcription—and, as such, they are an applicable model for the human disorder. To determine whether p53 deficiency alters the neoplastic phenotype of cartilage lesions, *Tg(Gli2;ColIIAI)* mice were crossed with mice expressing a null p53 allele (Jacks et al., 1994). The cartilage tumor phenotype was compared between *Tg(Gli2;ColIIAI);p53*$^{+/-}$ mice and littermate *Tg(Gli2;ColIIAI);p53*$^{+/+}$ mice. Since the metaphyseal cartilage lesions occurring in *Tg(Gli2;ColIIAI)* mice are observed at greatest frequency about the distal femur, we examined longitudinal sections of the bone for histological comparison. Three sections, equally distributed across the knee, were analyzed from each limb to allow for a systematic analysis. Cartilaginous lesions were identified in all of the mice. There were an average of 5.8 ± 2.3 lesions in the growth plate of *Tg(Gli2;ColIIAI);p53*$^{+/+}$ mice. These lesions measured under 0.5 mm in diameter (mean diameter, 0.32 ± 0.12 mm) and were composed of cells with occasional binucleate lacunae in a cartilage matrix. There were 12.3 ± 3.8 cells per high-powered field in the lesions (Figures 1A and 1C). Some of the lesions were connected to the growth plate through columns of cartilage. These lesions were identified in mice at all ages observed, up to 12 months of age.

In contrast, *Tg(Gli2;ColIIAI);p53*$^{+/-}$ mice developed larger (greater than 1 mm in diameter) cartilage lesions (mean diameter, 1.7 ± 0.3 mm) (Figures 1B and 1D). They were frequently in contact with the growth plate and had increased cellularity, compared to the smaller lesions (23.4 ± 7.8 cells per high-powered field). These cells showed variability in cytological appearance, seeming to have a broad range of characteristics of chondrocytes present during development (Figure 1F). There were pleiomorphic nuclei in many of the cells. These lesions were not observed in mice at 2 months of age, but were identified in a larger proportion of mice at subsequent time points. At 1 year of age, 50% of mice exhibited these lesions (Figure 1E). The appearance of the larger lesions did not change with the age of the mice (Figure 1E). Overall, this appearance is similar to that of a low-grade chondrosarcoma. We did not find a loss of the normal *p53* allele in these larger lesions, as determined using a PCR-based technique (Figure 1I). The level of *p53* expression was roughly half that of *Tg(Gli2;ColIIAI);p53*$^{+/+}$ littermates, suggesting normal expression from the wild-type allele (Figure 1J).

In a small number of mice (6%), a larger lesion (>1 cm in diameter) arose from bone, extending into the soft tissues. These lesions had a cytological appearance consistent with a higher grade sarcoma, composed of spindle-like cells (Figure 1G). These lesions showed loss of heterozygosity for the wild-type *p53* allele and exhibited a lack of *p53* RNA expression (Figures 1I and 1J).

p53 and Gli2 Interact to Regulate Chondrocyte Proliferation and ColX Expression in the Fetal Growth Plate

To determine whether the change in phenotype was due to alterations in proliferation or differentiation, we examined these parameters in the tumors and distal femurs of fetal limbs. Proliferation was examined in the tumors using phosphohistone H3 staining, showing a trend toward a greater percentage of stained cells in the larger lesions that developed in the *Tg(Gli2;ColIIAI);p53*$^{+/-}$ mice, compared with lesions in *Tg(Gli2;ColIIAI);p53*$^{+/+}$ mice. There was also little difference in the proportion of cells staining for ColX. We examined these same parameters in the growth plate from mature mice and also did not observe substantial differences. Since changes in chondrocyte behavior that could be responsible for the changes in the tumor phenotype may be more readily apparent in fetal limbs, we examined