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Materials and Methods

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Sustained Loss of a Neoplastic Phenotype by Brief Inactivation of *MYC*

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Pharmacological inactivation of oncogenes is being investigated as a possible therapeutic strategy for cancer. One potential drawback is that cessation of such therapy may allow reactivation of the oncogene and tumor regrowth. We used a conditional transgenic mouse model for *MYC*-induced tumorigenesis to demonstrate that brief inactivation of *MYC* results in the sustained regression of tumors and the differentiation of osteogenic sarcoma cells into mature osteocytes. Subsequent reactivation of *MYC* did not restore the cells' malignant properties but instead induced apoptosis. Thus, brief *MYC* inactivation appears to cause epigenetic changes in tumor cells that render them insensitive to *MYC*-induced tumorigenesis. These results raise the possibility that transient inactivation of *MYC* may be an effective therapy for certain cancers.

Activation of oncogenes plays an important role in tumorigenesis (*1*). Strategies that inactivate oncogenes for the treatment of cancer are in development; however, such approaches may be limited by the toxicity caused by the prolonged inactivation of the associated proto-oncogene. Moreover, cessation of the pharmacologic inactivation of an oncogene may result in tumor regrowth. To determine whether brief oncogene inactivation can produce sustained tumor regression, we used the tetracycline regulatory system to conditionally regulate *MYC* expression in transgenic mice. We previously described transgenic mice that conditionally express *MYC* in their lymphocytes (*2*). About 1% of these mice develop osteogenic sarcomas, and these tumors expressed abundant levels of MYC, presumably because the EμSRα enhancer causes *MYC* expression in immature osteoblasts. Consistent with this, *MYC* is commonly overexpressed in human and rodent osteogenic sarcomas (*3–11*).

The tumors in our transgenic model share some features with human osteogenic sarcoma (*12–14*). They present as invasive masses in the skeleton; they are associated with disorganized bone matrix; and they readily metastasize (fig. S1) (*15*). These properties were maintained as the tumors were adapted to in vitro growth and were inoculated into syngeneic hosts (*15*). To investigate the effects of *MYC* inactivation, we administered doxycycline (dox) treatment to mice with transplanted osteogenic sarcoma cells or primary transgenic tumors. After dox treatment in vivo, osteogenic sarcomas stopped expressing the *MYC* transgene, differentiated into mature bone, and exhibited sustained tumor regression (fig. S2). Similarly, primary transgenic tumors regressed and differentiated into bone (fig. S4). After dox treatment in vitro, the tumor cells exhibited a reduced growth rate, assumed a flattened morphology, lost alkaline phosphatase activity, and continued to express osteopontin (*15*) (Fig. 1). These phenotypic features are associated with the differentiation of immature osteoblasts into mature osteocytes (*7*, *16–18*). We conclude that *MYC* inactivation causes osteogenic sarcoma cells to differentiate into mature osteocytes.

To examine the effects of *MYC* inactivation and reactivation in individual tumor cells, we cultured osteogenic sarcoma cells

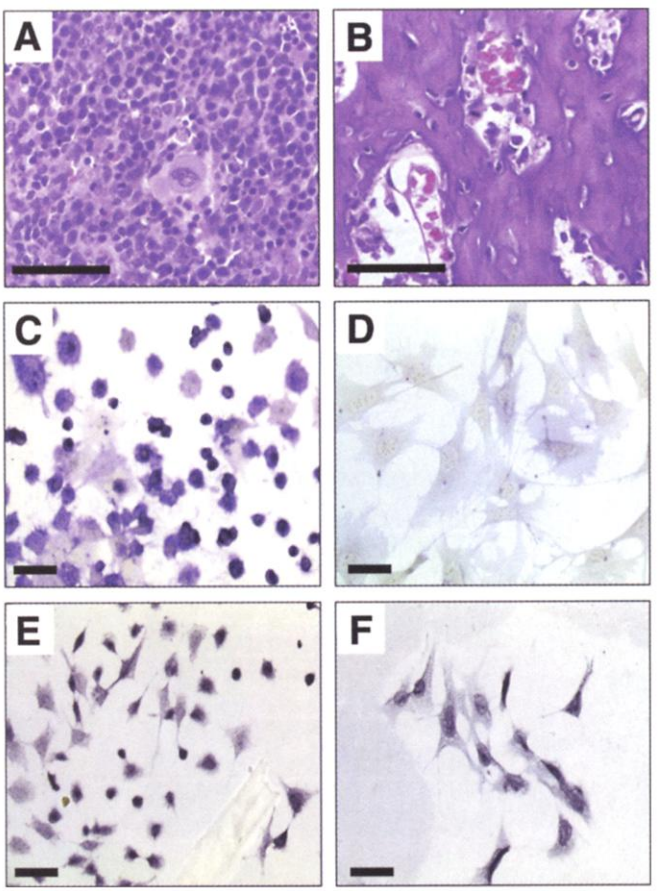

Fig. 1. Inactivation of *MYC* causes regression and differentiation of tumor cells. *MYC* inactivation resulted in the differentiation of **(A)** osteogenic sarcomas into **(B)** mature osteoid. Alkaline phosphatase activity **(C)** before and **(D)** after dox treatment. Osteopontin expression **(E)** before and **(F)** after dox treatment. Representative data from one of five experiments. At least five mice were injected per experiment. Similar results were seen for two other transplanted tumors and two independent primary transgenic tumors (*15*). Bars, 50 μm.

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in vitro and analyzed them by continuous video time-lapsed (CVTL) microscopy (*15*). Before treatment, the tumor cells rapidly proliferated, undergoing cell division every 14 hours; however, within 24 hours of *MYC* inactivation, the tumor cells flattened (fig. S3) and showed one-tenth as much cell division (Fig. 2). This activity is consistent with the differentiation of immature osteogenic sarcoma cells into mature osteocytes. Next, we reactivated *MYC* expression in the cultured cells by withdrawing dox treatment. In contrast to our prediction that the tumor cells would resume proliferation, the tumor cells underwent apoptosis in a stochastic manner over a period of 48 hours, and total cell numbers were reduced (Fig. 2). About 5% of the tumor cells did not undergo apoptosis but retained the morphology of mature osteocytes. Less than 1% of the tumor cells regained their neoplastic growth properties.

To examine whether *MYC* reactivation had similar effects in vivo, we transplanted osteogenic sarcomas subcutaneously into

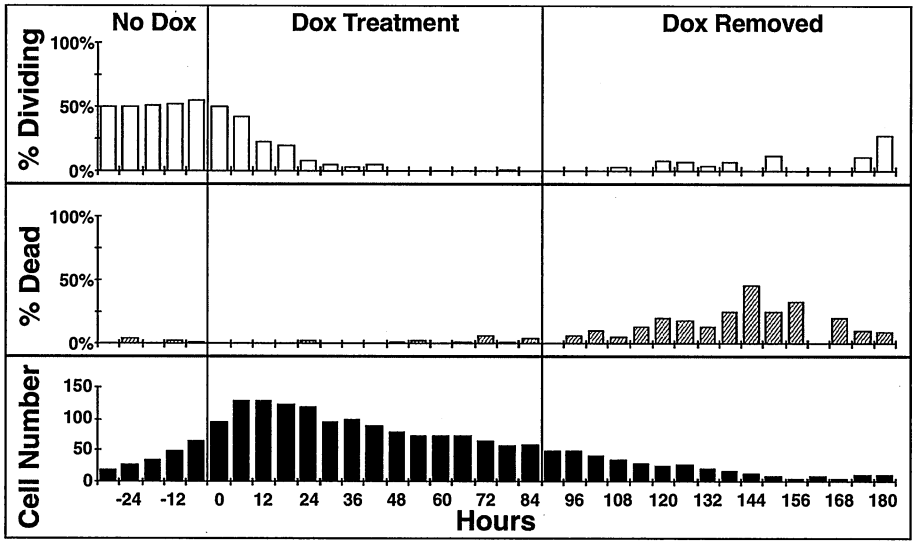

Fig. 2. *MYC* inactivation followed by reactivation in vitro causes apoptosis of tumor cells. The percentages of dividing cells and dying cells and the total number of cells were recorded for each 6-hour interval. Individual cells that remained in nine different fields were observed by CVTL microscopy during each interval. The loss of cells observed after dox treatment was not because of cell death but rather the inability to follow these cells by CVTL microscopy due to cell migration out of the observed field. Representative data are shown from one of two experiments. Similar results were observed for two additional osteogenic sarcoma cell lines by conventional microscopy.

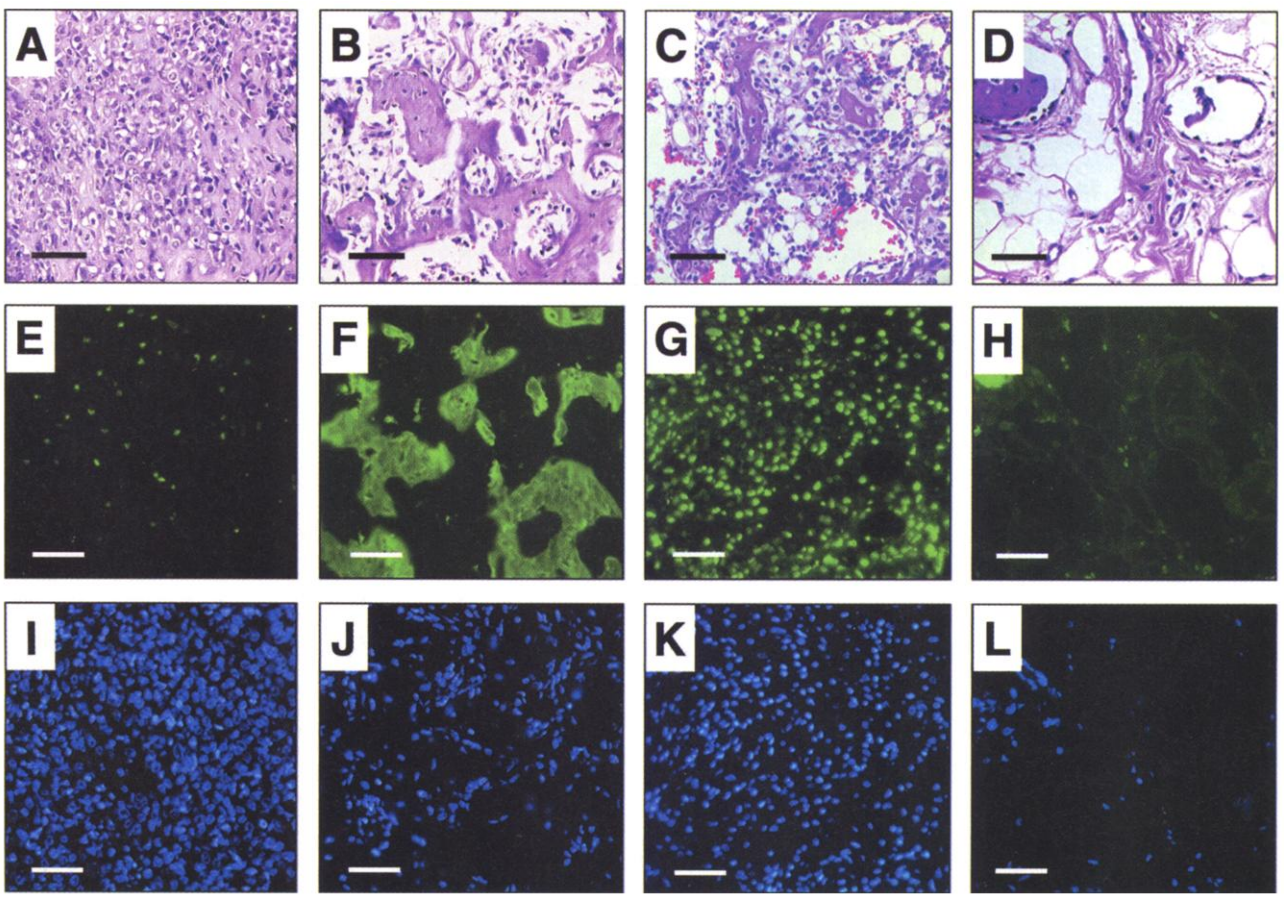

Fig. 3. *MYC* inactivation followed by reactivation in vivo causes apoptosis of tumor cells. Tumor cells were transplanted subcutaneously into syngeneic mice. When tumors reached a diameter of 0.5 to 1.0 cm, mice were killed (*MYC*-activated) or treated with dox for 10 days (*MYC*-inactivated). Dox treatment was terminated (*MYC*-reactivated), and the mice were examined either 5 or 14 days later. The bone matrix exhibited a high degree of autofluorescence. (**A** to **D**) Hematoxylin and eosin staining, (**E** to **H**) TUNEL staining, and (**I** to **L**) DAPI staining. (A), (E), and (I), *MYC*-activated; (B), (F), (J), *MYC*-inactivated; (C), (G), (K), *MYC*-reactivated for 5 days; and (D), (H), (L), *MYC*-reactivated for 14 days. Bars, 50 μm. Representative results are shown from one of two experiments, each with about six mice. Similar results were seen in two independent primary osteogenic sarcomas (*15*).

syngeneic mice, allowed the tumors to grow from 0.5 to 1.0 cm in diameter, and then administered dox treatment for 10 days. As before, this treatment induced the differentiation of tumor cells into osteocytes. After 10 days, we reactivated *MYC* by terminating dox treatment. No histological changes were evident after 5 days; however, at 14 days after *MYC* reactivation there was a marked reduction in the total number of tumor cells (Fig. 3, A to D). To determine whether the tumor cells were dying by apoptosis, we performed a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay on formalin-fixed, paraffin-embedded sections (*15*). The tumors exhibited a low frequency of background apoptosis before and after *MYC* inactivation (Fig. 3, E and F). In contrast, at 5 days after *MYC* reactivation, differentiated tumor cells exhibited a marked increase in apoptosis (Fig. 3G). At 14 days after *MYC* reactivation, only rare TUNEL positive cells were observed, most likely because most of the cells had already undergone apoptosis (Fig. 3H). The 4′,6′−diamidino-2-phenylindole (DAPI) staining of the same specimens confirmed the presence of a high density of tumor cells (Fig. 3I), a lower density of differentiated tumor cells as compared to the amount present after *MYC* inactivation (Fig. 3J), a reduced number of tumor cells 5 days after *MYC* reactivation (Fig. 3K), and nearly a complete absence of tumor cells 14 days after *MYC* reactivation (Fig. 3L). Upon *MYC* reactivation, we did not observe evidence for tumor regrowth. Similarly, in primary transgenic tumors, the inactivation of *MYC* was associated with the differentiation of tumors into bone, and the reactivation of *MYC* was associated with the apoptosis of tumor cells (fig. S4).

We then investigated whether temporary loss of *MYC* overexpression causes the irreversible loss of a tumorigenic phenotype (Fig. 4). We intraperitoneally injected syngeneic mice with tumor cells and treated them 2 days later with dox to inactivate *MYC*. Untreated mice succumbed to tumors within 5 weeks, whereas dox-treated mice survived more than 20 weeks. Mice treated with dox for only 10 days were free of tumors for more than 12 weeks after the cessation of treatment, and 2 of 12 mice in this group survived for 20 weeks. When the tumors in these mice began to form, readministration of dox treatment resulted in the regression of two of the tumors tested. In addition, dox treatment differentiated the tumor cells taken from mice that had succumbed to neoplasia in five of five tumors tested.

We conclude that although oncogene-induced tumorigenesis is reversible (*2*, *19*–*21*), the reactivation of an oncogene does not necessarily restore a neoplastic phenotype. Our observation that *MYC* reactivation induces tumor cell apoptosis conflicts with previous studies showing that *MYC* is tumorigenic only in cells unable to undergo apoptosis (*22*). Our results also conflict with earlier findings that transient *MYC* activation can induce tumorigenesis in rodent immortal cell lines (*23*). One possible explanation is that the effects of *MYC* inactivation and reactivation may depend on the mechanism by which *MYC* contributes to tumorigenesis, which is likely to vary according to the genetic and cellular context. When *MYC* causes tumorigenesis by promoting cellular proliferation and blocking cellular differentiation, its inactivation results in proliferative arrest and differentiation of tumor cells as well as the loss of the neoplastic properties, as described here and previously for hematopoietic tumors (*2*). By contrast, when *MYC* causes tumorigenesis by promoting genomic destabilization, its inactivation would not result in tumor regression (*23*).

We speculate that tumors arise as a result of a combination of genetic events that occur in a requisite epigenetic context (*24*). There may be precise opportunities during differentiation that provide the permissive context in which oncogene activation produces a neoplastic phenotype. The brief inactivation of an oncogene can change this epigenetic context, thereby revoking its ability to maintain tumorigenesis. If valid, this model could have important implications for the development of new cancer drugs. Long-term use of drugs that are designed to inactivate oncogenes would be expected to have serious toxicities because they also disrupt critical signaling pathways in normal cells. Our results suggest that it may be possible to briefly inactivate oncogenes in the treatment of cancer, thereby mitigating toxicities without compromising the efficacy of such therapy. We recognize that in human tumors it may be more difficult to induce the regression of cancers through oncogene inactivation (*25*).

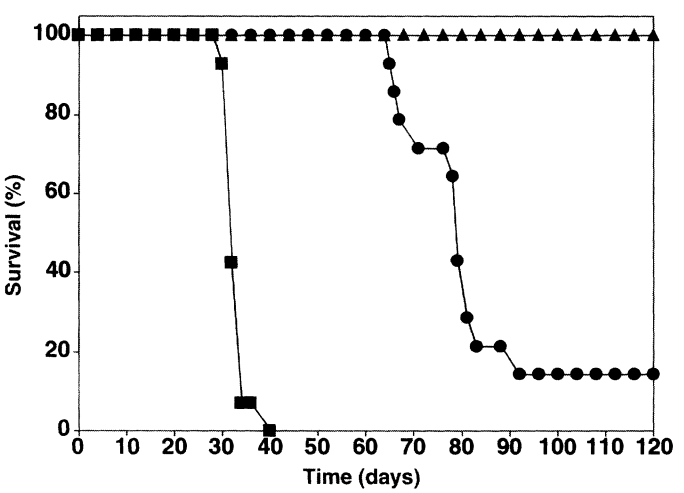

Fig. 4. Transient inactivation of *MYC* transgene can increase the survival of syngeneic mice that received tumor transplants. Tumor cells (1×10^5) were injected intraperitoneally. Two days after injection of tumor cells, mice were left untreated (■), treated with dox continuously (▲), or treated with dox for only 10 days (●). Mice were killed when moribund with tumor burden. Three independent experiments were performed with one tumor cell line. The graph represents pooled data from all experiments, each with at least 14 mice per group.

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Figs. S1 to S4

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