Establishment of a Dog Model for the p53 Family Pathway and Identification of a Novel Isoform of p21 Cyclin-Dependent Kinase Inhibitor

Jin Zhang, Xiangling Chen, Michael S. Kent, Carlos O. Rodriguez, and Xinbin Chen

Comparative Cancer Center, University of California, Davis, California

Abstract

Spontaneous tumors in the dog offer a unique opportunity as models to study human cancer etiology and therapy. *p53*, the most commonly mutated gene in human cancers, is found to be altered in dog cancers. However, little is known about the role of p53 in dog tumorigenesis. Here, we found that on exposure to DNA damage agents or MDM2 inhibitor nutlin-3, canine p53 is accumulated and capable of inducing its target genes, *MDM2* and *p21*. We also found that on DNA damage, canine p53 is accumulated in the nucleus, followed by MDM2 nuclear translocation and increased 53BP1 foci formation. In addition, we found that canine p63 and p73 are up-regulated by DNA damage agents. Furthermore, colony formation assay showed that canine tumor cells are sensitive to DNA damage agents and nutlin-3 in a p53-dependent manner. Surprisingly, canine p21 is expressed as two isoforms. Thus, we generated multiple canine p21 mutants and found that amino acids 129 to 142 are required, whereas amino acid 139 is one of the key determinants, for the expression of two p21 isoforms. Finally, we showed that although the full-length human p21 cDNA expresses one polypeptide, amino acid 139 seems to play a similar role as that in canine p21 for various migration patterns. Taken together, our results indicate that canine p53 family proteins have biological activities similar to human counterparts. These similarities make the dog an excellent outbred spontaneous tumor model, and the dog can serve as a translation model from benchtop to cage side and then to bedside. (Mol Cancer Res 2009;7(1):67–78)

Introduction

The anatomic and physiologic similarities between dogs and humans have been the basis of using the dog in biomedical research for over a century. Recently, the spontaneously occurring tumors in companion dogs have become an ideal model for cancer research when compared with the murine model owing to the following features: (*a*) Dog cancers occur spontaneously, whereas in laboratory mice cancers are mostly induced by carcinogens and/or genetic manipulations; (*b*) dog tumors, including melanoma, osteosarcoma, lung carcinoma, head and neck carcinoma, and mammary carcinoma, have similar histologic appearance and response to conventional chemotherapies as human tumors; (*c*) humans and companion dogs live in the same environment; and (*d*) genetically, dogs are closer to humans than mice, including many oncogenes and tumor suppressor genes, which are found to be altered in spontaneous human tumors as well as in spontaneous dog tumors.

The p53 tumor suppressor plays a pivotal role in preserving the integrity of the genome and in maintaining normal cell cycle regulation. Mutations of p53 tumor suppressor occur in ~50% of human cancers and loss of p53 function is known to play a central role in cancer development and progression (1). p53 is expressed at low levels under unperturbed conditions and rapidly stabilized under stress conditions (1). p53 functions as a transcriptional factor to transactivate its downstream targets involved in cell cycle arrest and apoptosis. Thus, p53 and its downstream targets consist of a network, where p53 is a key molecular node in the network. In the late 1990s, two p53-related genes, *p63* and *p73*, were identified due to the high degree of sequence similarity, especially in the DNA-binding domain (2, 3). As a result, p63 and p73 can transactivate some p53-responsive genes involved in cell cycle arrest and apoptosis. Thus, these three proteins form the p53 family. Interestingly, the activities of p53 family proteins are not entirely redundant because p53, p63, and p73 knockout mice exhibit distinct phenotypes (4-7), indicating that each p53 family member has its own specific biological functions.

The canine *p53* gene shares 87% sequence similarity with the human *p53* gene according to the National Center for Biotechnology Information d8atabase. Like its human counterpart, canine p53 is found to be mutated in several types of dog tumors, including osteosarcoma (8), mammary tumors (9), and mastocytoma (10). However, little is known about the biological function of canine p53 and its role in dog tumorigenesis, and even less is known about the other two p53-related genes, *p63* and *p73*. In the present study, we have explored the role of p53 and its family members, p63 and p73,

Received 7/22/08; revised 9/5/08; accepted 10/2/08.
Grant support: NIH grants CA076069, CA081237, and CA102188 (X. Chen) and Cancer Center Support grant CA093373 to University of California Davis Cancer Center.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).
J. Zhang and X. Chen contributed equally to this work.
Requests for reprints: Xinbin Chen, VM: Surgical and Radiological Sciences, Tupper 2128, University of California, Davis, CA 95616. Phone: 530-754-8404; Fax: 530-752-6042. E-mail: xbchen@ucdavis.edu

doi:10.1158/1541-7786.MCR-08-0347

in canine normal and tumor cell lines. We found that DNA damage agents, such as camptothecin and doxorubicin, are capable of up-regulating all three p53 family members as well as p53 targets, p21 and MDM2, whereas the MDM2 inhibitor nutlin-3 is only capable of up-regulating p53. Immunofluorescence assay showed that canine p53 is accumulated in the nucleus on DNA damage, which is coupled with MDM2 nuclear translocation and increased 53BP1 foci formation. In addition, we found that canine tumor cells are sensitive to DNA damage agents and nutlin-3 in a p53-dependent manner. Finally, we showed that canine p21 is expressed as two isoforms and the region of amino acids 129 to 142 is required for the formation of two isoforms.

Results

The Expression Pattern of p53 in Canine Cell Lines

To explore canine p53 expression, five melanoma cell lines (melanoma-12, melanoma-23, melanoma-36, melanoma-50, and melanoma-64) and four osteosarcoma cell lines (D17, 48-4, 348617, and 340529) were used. Some of the cell lines have been characterized to be tumorigenic in nude mice (11). In addition, Madin-Darby canine kidney (MDCK) and Cf2Th thymus cell lines were purchased from American Type Culture Collection (ATCC) for this study. MDCK cell line was derived from a normal canine kidney and known to contain wild-type p53 (12), whereas Cf2Th cell line was derived from a normal canine thymus tissue. These canine cells were mock treated or treated with camptothecin, doxorubicin, or nutlin-3 for 12 hours and the levels of dog p53 protein were measured by Western blotting. Both camptothecin and doxorubicin are DNA damage agents, which can cause DNA double-strand breaks and are known to stabilize p53 in human cells (13), whereas nutlin-3 is an MDM2 inhibitor and prevents MDM2-mediated p53 degradation (14). We found that canine p53 was accumulated in MDCK cells in response to camptothecin, doxorubicin, or nutlin-3 (Fig. 1A, *p53*, compare *lanes 2-4* with *lane 1*), consistent with a previous report (12). Likewise, p53 was induced in four melanoma cell lines (melanoma-12, melanoma-23, melanoma-50, and melanoma-64) on treatment with camptothecin, doxorubicin, and nutlin-3 (Fig. 1A, *p53*, compare *lanes 6-8, 10-12*, and *14-16* with *lanes 5, 9*, and *13*, respectively; Fig. 1B, *p53*, compare *lanes 6-8* with *lane 5*). However, p53 was undetectable in one melanoma cell line (melanoma-36) regardless of treatment (Fig. 1B, *p53, lanes 9-12*), suggesting that melanoma-36 is likely to be p53 null. To determine whether the stabilized p53 in these cells is transcriptionally active, we measured induction of two well-known p53 targets, p21 and MDM2. p21 is required for p53-mediated G_1 arrest (15) whereas MDM2 is an E3 ubiquitin ligase and can target p53 for proteasomal degradation (16, 17). We showed that in response to camptothecin, doxorubicin, and nutlin-3, both p21 and MDM2 were induced in cells with wild-type p53, including MDCK and four melanoma cell lines, but not in the p53-deficient melanoma-36 cell line (Fig. 1A and B, *p21* and *MDM2*). Surprisingly, canine p21 is expressed as two isoforms (Fig. 1A and B, *p21*), which is addressed in the latter part of this study.

Next, we analyzed the induction of p53, p21, and MDM2 in Cf2Th and four osteosarcoma cell lines (D17, 48-4, 348617, and 340529). We found that all osteosarcoma cells showed a marked accumulation of p53 in response to camptothecin, doxorubicin, and nutlin-3 (Fig. 1C and D, *p53*, compare *lanes 6-8* and *10-12* with *lanes 5* and *9*, respectively). In addition, p21 was induced (Fig. 1C and D, *p21*, compare *lanes 6-8* and *10-12* with *lanes 5* and *9*, respectively). However, MDM2 was highly induced in cells treated with nutlin-3 but little, if any, with camptothecin or doxorubicin (Fig. 1C and D, *MDM2*, compare *lanes 9* and *12* with *lanes 7-8* and *10-11*, respectively). Surprisingly, the steady-state level of p53 protein was found to be very high in Cf2Th cells (Fig. 1D, *p53*, compare *lane 1* with *lanes 5* and *9*). In addition, p53, p21, and MDM2 were not found to be induced in Cf2Th cells on treatment with camptothecin, doxorubicin, or nutlin-3 (Fig. 1D, *p53, p21*, and *MDM2*, compare *lanes 2-4* with *lane 1*). Because Cf2Th cell line was derived from a normal canine thymus tissue, it was assumed to be a nontumor cell line. However, our results suggest that Cf2Th carries a mutant p53 and thus is likely to be a tumor cell line.

The Intracellular Localization of p53, MDM2, and 53BP1 in MDCK Cells

In response to genotoxic stress, human p53 is accumulated in the nucleus to transactivate its downstream targets. Thus, we sought to determine the intracellular localization of canine p53 in response to doxorubicin by immunofluorescence assay. To do so, MDCK cells were mock treated or treated with doxorubicin for 12 hours and then stained with anti-p53 and 4′,6-diamidino-2-phenylindole (DAPI). We found that in the absence of doxorubicin, there was little positive staining of p53 (Fig. 2A, *top*), whereas in the presence of doxorubicin, condensed nuclear staining of p53 was observed (Fig. 2A, *bottom*). In addition, we analyzed the intracellular localization of MDM2. As a p53 target, it is expected that activation of p53 on DNA damage would lead to increased expression of MDM2. Indeed, we found that in the absence of DNA damage, MDM2 was weakly stained and primarily localized in the cytoplasm (Fig. 2B, *top*). However, on treatment with doxorubicin, the intensity of MDM2 was markedly increased (Fig. 2B, *bottom*). Most importantly, the vast majority of MDM2 protein was translocalized from the cytoplasm into the nucleus in response to DNA damage (Fig. 2B). To further test this, we analyzed the localization of 53BP1, which is known to interact with p53 and functions as a modulator of the p53 and DNA damage response pathways (18). We found that in response to DNA damage, a large number of 53BP1 foci were detected in the nucleus as compared with that under the control condition (Fig. 2C), consistent with previous observations in human cells (19).

The Expression Patterns of p63 and p73 in Canine Cells

Human p63 and p73 have been found to play a critical role in tumor suppression and normal development (5-7). However, there has been little study of canine p63 and p73. Based on the predicted sequence, canine p63 and p73 share 99.6% and 81% sequence identity with their human homologues, respectively. Thus, canine p63 and p73 might be recognized by commercial available antibodies against human p63 and p73, respectively.

In this regard, canine p63 was detected by Western blotting with 4A4 anti-p63 antibody. 4A4 was raised against amino acids 1 to 205 in ΔNp63α of human origin and is capable of recognizing both TA and ΔN p63 isoforms in humans. We found that several distinct bands were detected in MDCK cells treated with camptothecin or doxorubicin compared with that in mock-treated MDCK cells (Fig. 3A, *p63*, compare *lanes 2* and *3* with *lane 1*), suggesting that these are likely to represent various TA and/or ΔN p63 isoforms. However, whereas nutlin-3 was able to stabilize p53 in dog cells (Fig. 1), the levels of these p63 proteins were not significantly altered (Fig. 3A, *p63*, compare *lane 4* with *lane 1*). We also found that some of the p63 isoforms were increased in melanoma and osteosarcoma cells on treatment with camptothecin and doxorubicin, but not with nutlin-3 (Fig. 3A-C), consistent with the observations in human cells (20). Interestingly, in melanoma-36 and D17 cells, one isoform of p63 was found to be highly expressed (Fig. 3B, *lanes 9-12*; Fig. 3C, *lanes 5-8*).

Next, we determined the expression pattern of p73 in canine cells using BL906 antibody. BL906 was raised against amino acids 1 to 62 in human TAp73 and can only detect various TAp73 isoforms. We noticed that based on the expression pattern and molecular mass, the most predominant isoform in canine cells is likely to represent TAp73α (Fig. 4, marked by *dot*). We also found that the predominant TAp73 isoform was markedly induced in cells on treatment with camptothecin and doxorubicin, but not nutlin-3 (Fig. 4A-C). In addition, in melanoma-36 and D17 cells, this isoform of TAp73 was found to be highly expressed (Fig. 4B and C). In sum, our results indicate that, like their human counterparts, canine p63 and p73 can be up-regulated in cells on DNA damage, but not by MDM2 inhibitor nutlin-3.

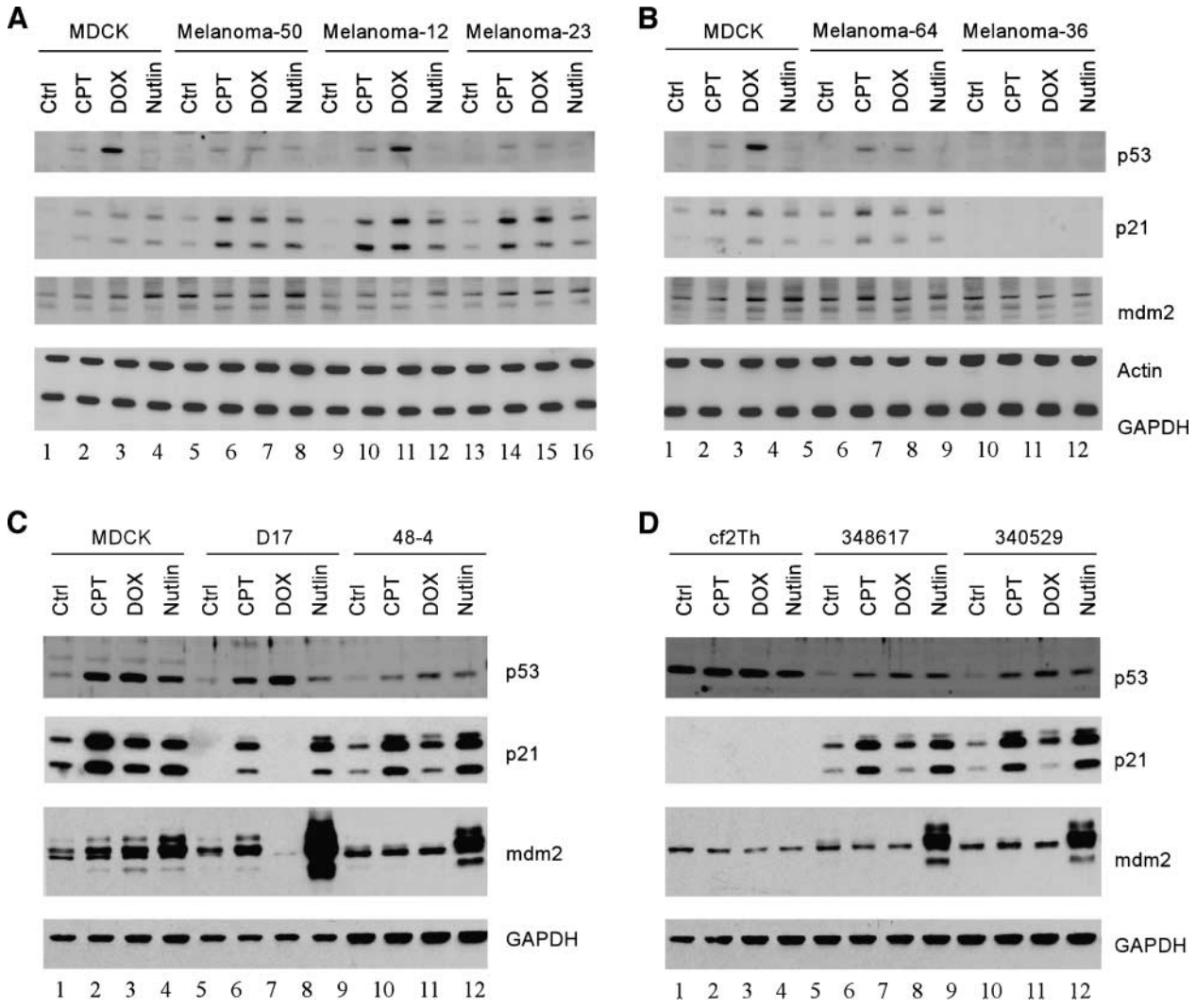

FIGURE 1. The expression pattern of p53 in canine cell lines. **A** and **B.** p53 is induced by DNA damage agents or nutlin-3 in MDCK and four melanoma (melanoma-50, melanoma-12, melanoma-23, and melanoma-64) cells but not in melanoma-36 cells. Cells were treated with or without camptothecin (*CPT*), doxorubicin (*DOX*), or nutlin-3 for 12 h. Cell lysates were collected and subjected to Western blot analysis with antibodies against p53, p21, MDM2, actin, and GAPDH. **C** and **D.** p53 is induced in four osteosarcoma cells (D17, 48-4, 348617, and 340529) but not in Cf2Th cells. Cells were treated as described in **A** and then analyzed by Western blot analysis with antibodies against p53, p21, MDM2, and GAPDH.

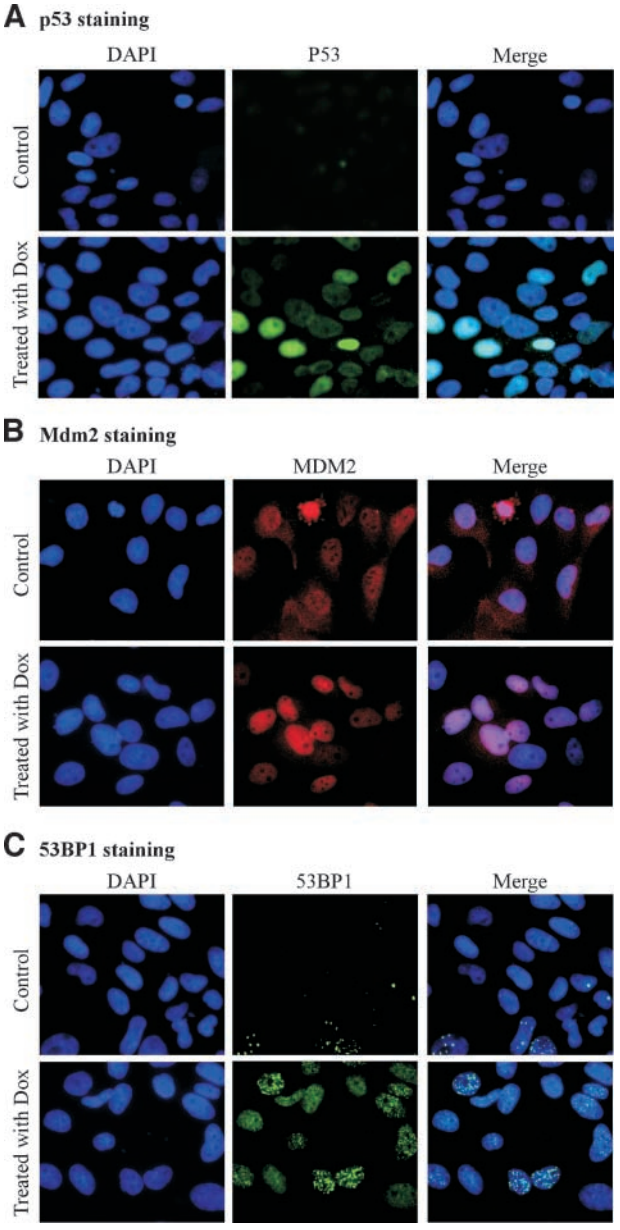

FIGURE 2. The intracellular localization of p53, MDM2, and 53BP1 in MDCK cells. **A.** p53 is accumulated in the nucleus of MDCK cells on doxorubicin treatment. MDCK cells were mock treated or treated with doxorubicin (250 ng/mL) for 12 h. Cells were then fixed and stained for p53 and DAPI. The nuclear DAPI stain is blue and p53 stain is green. **B.** MDM2 is translocated from the cytoplasm to the nucleus in response to DNA damage. Cells were treated as described in **A**, followed by immunofluorescence assay using anti-MDM2 and DAPI. **C.** 53BP1 foci formation is increased in response to DNA damage. Cells were treated as described in **A**, followed by immunofluorescence assay using anti-53BP1 and DAPI.

The Sensitivity of Canine Cells to DNA Damage Agents and MDM2 Inhibitor Nutlin-3

One of the most important functions of the p53 family proteins is to suppress cell growth (21). Thus, we determined if nutlin-3, which can induce canine p53 expression, has an effect on dog cell proliferation. To test this, cells were mock treated or treated with 0.25, 0.5, or 1 μmol/L of nutlin-3, and then grown for 10 days. Colony formation assay showed that on nutlin-3 treatment, the ability of the cells to form colonies was markedly inhibited in a dosage-dependent manner in MDCK and four melanoma cell lines (melanoma-12, melanoma-23, melanoma-50, and melanoma-64; Fig. 5A and B; Supplementary Fig. S1). It should be noted that in these cell lines, endogenous p53 is likely to be wild-type (Fig. 1A and B). However, melanoma-36 cells, in which endogenous p53 was undetectable and likely to be lost (Fig. 1B), were insensitive to nutlin-3 (Fig. 5C). Next, the sensitivity of canine cells to chemotherapeutic agents, camptothecin and doxorubicin, was similarly examined. We found that both camptothecin and doxorubicin greatly inhibited colony formation in MDCK and all the melanoma cells (Fig. 6A-C; Supplementary Fig. S2) although melanoma-36 cells were slightly less sensitive to camptothecin (Fig. 6C). Similarly, the sensitivity of Cf2Th and the four osteosarcoma cell lines to nutlin-3, camptothecin, and doxorubicin was examined by colony formation assay. MDCK cell line was used as a control and found to be sensitive to nutlin-3 (Fig. 7A) as detected above (Fig. 5A). We found that the ability of osteosarcoma cells to form colony was inhibited on treatment with nutlin-3 in a dose-dependent manner (Fig. 7B and C; Supplementary Fig. S3), consistent with the possibility that these osteosarcoma cell lines carry wild-type p53 (Fig. 1C and D). In addition, three osteosarcoma cell lines (340529, 48-4, and 348617) were highly sensitive to camptothecin and doxorubicin (Fig. 8C; Supplementary Fig. S4) whereas D17 osteosarcoma cell line was sensitive to doxorubicin but not camptothecin (Fig. 8D). However, Cf2Th cell line was insensitive to nutlin-3 (Fig. 7D), camptothecin (Fig. 8B), and doxorubicin (Fig. 8B), consistent with the notion that Cf2Th carries a mutant p53 (Fig. 1D).

Identification of a Novel Isoform of Canine p21 and Possibly Human p21

As we showed above, canine p21 is expressed as two isoforms (Fig. 1) whereas human p21 is mainly expressed as one protein (15). To address why this occurs, we first sought to clone and express the canine *p21* gene. According to the National Center for Biotechnology Information database, both human and canine *p21* genes contain three exons. Nucleotide sequence analysis showed that the first exon is not conserved whereas the second and third exons are highly conserved between humans and dogs. However, the predicted second exon in the dog lacks the first 45 nucleotides along with an ATG initiation codon, compared with that in human p21. In this regard, the canine genomic DNA sequence was searched for an additional sequence homologous to the human p21. Indeed, a highly conserved p21 polypeptide was deduced from the dog genomic DNA sequence and shown in Fig. 9. Like human p21, canine p21 protein also contains 164 amino acids (Fig. 9). Canine p21 protein shares 82% amino acid identity to human p21, compared to 76% sequence identity between mouse and human p21 (Fig. 9). Based on the p21 genomic DNA sequence, a pair of primers was designed to amplify the canine p21-coding sequence by reverse transcription-PCR with total RNA purified from MDCK cells. Next, to make sure that the amplified canine p21 cDNA truly

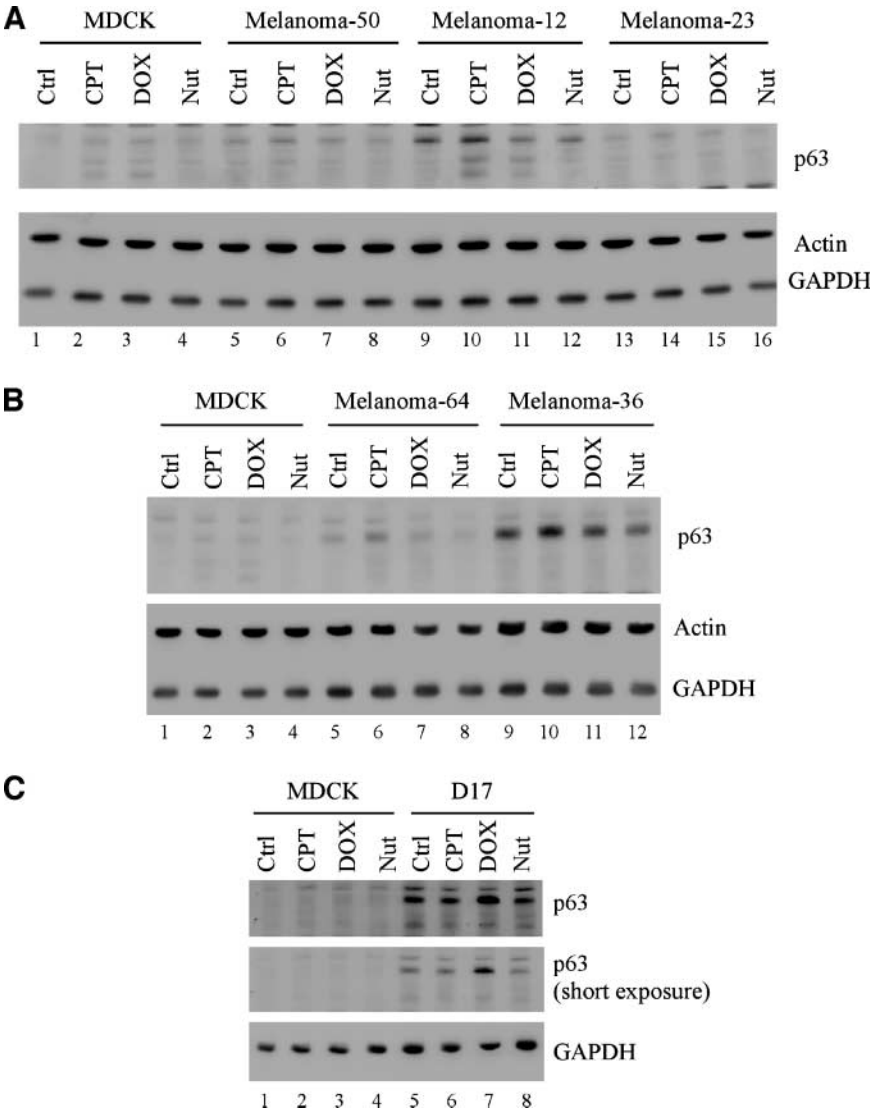

FIGURE 3. The expression pattern of p63 in canine cell lines. **A** and **B.** Expression of various p63 isoforms in MDCK and melanoma cells mock treated or treated with camptothecin, doxorubicin, or nutlin-3 for 12 h. Cell lysates were collected and subjected to Western blot analysis with antibodies against p63, actin, and GAPDH. **C.** Expression of various p63 isoforms in MDCK and D17 cells mock treated or treated with camptothecin, doxorubicin, or nutlin-3 for 12 h. The experiment was done as described in **A** and **B**.

represents the canine *p21* gene, the cDNA was cloned into pCDNA3 expression vector and then expressed in both human RKO and canine MDCK cells. We found that like endogenous p21 in MDCK cells, two p21 isoforms were expressed by canine p21 cDNA and had the same migration patterns as endogenous dog p21 in both human and canine cells (Fig. 10A, *p21*, compare *lanes 3* and *5* with *lane 4*). This suggests that the dog *p21* gene, but not the cell type, is responsible for p21 expression pattern. We would also like to note that the predominant form of human p21 detected in RKO cells migrated between the two dog p21 isoforms (Fig. 10A, compare *lanes 1* and *2* with *lane 3*).

Next, to determine if the two isoforms of dog p21 are due to alternative translation start site, p21(38-164), an NH_2-terminally truncated p21 was cloned, which begins at codon 38, a potential ATG initiation codon (Fig. 10B). Both canine full-length p21 cDNA and canine p21(38-164) were transfected into Cf2Th cells because endogenous p21 in Cf2Th cells was found to be undetectable (Fig. 1D, *lanes 1-4*). Like the expression pattern in RKO and MDCK cells (Fig. 10A), canine p21 was expressed as two isoforms (Fig. 10C, *lane 2*). In addition, the NH_2-terminally truncated p21(38-164) was also expressed as two isoforms (Fig. 10C, *lane 3*), suggesting that expression of two p21 isoforms is not due to the usage of alternative translation start codons. On the other hand, it suggests that the region of amino acids 1 to 37 is not responsible for the expression of two p21 isoforms in the dog.

To further characterize the region in the dog *p21* gene responsible for the expression of two isoforms, various deletion mutants of canine p21 cDNAs were generated (Fig. 10B), including p21(1-152), p21(1-142), p21(1-129), p21(1-121), and p21(1-93). To facilitate protein detection, canine p21 was tagged with a hemagglutinin (HA) epitope at its NH_2 terminus (Fig. 10B). We found that two isoforms were expressed from mutant p21(1-152) and p21(1-142), but not p21(1-129), p21 (1-121), and p21(1-93) (Fig. 10D). Interestingly, we noticed that the p21 mutant (1-129) migrated between the two isoforms

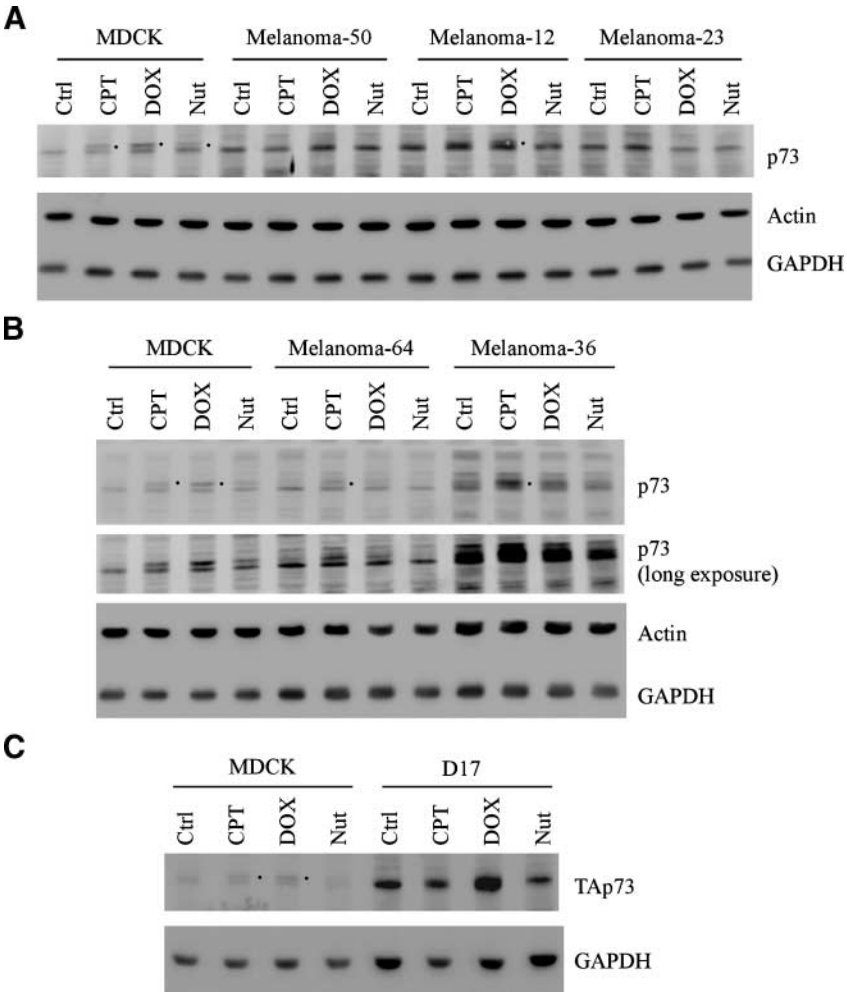

FIGURE 4. The expression pattern of p73 in canine cell lines. **A** and **B.** Expression of TAp73 in MDCK and melanoma cells mock treated or treated with camptothecin, doxorubicin, or nutlin-3 for 12 h. Cell lysates were collected and subjected to Western blot analysis with antibodies against p73, actin, and GAPDH. **C.** Expression of TAp73 in MDCK and D17 cells mock treated or treated with camptothecin, doxorubicin, or nutlin-3 for 12 h. The experiment was done as described in **A** and **B**.

of the mutant p21(1-142) although it is 13 amino acids less (Fig. 10D, compare *lane 4* and *5*). These results suggest that the region of amino acids 129 to 142 is responsible for the expression of two p21 isoforms in the dog. To further delineate the region of amino acids 129 to 142, additional deletion mutants of canine p21 cDNAs were generated (Fig. 10E), including p21(1-141), p21(1-140), p21(1-139), p21(1-138), and p21(1-136). We found that all p21 mutants were expressed as two isoforms (Fig. 10F, *left*). However, the expression patterns of the two isoforms were different among these mutants. The difference in migration between the two isoforms expressed by p21(1-152), p21(1-142), p21(1-141), and p21(1-140) was much greater than that expressed by p21(1-139), p21(1-138), and p21(1-136), suggesting that amino acid 139 is one of the key determinants for the expression of two p21 isoforms. Due to the remarkable sequence similarity between dog and human p21, we examined whether human p21 has similar patterns in migration. To address this, multiple human p21 deletion mutants, which correspond to the canine p21 mutants, were generated (Fig. 10E). We found that although the full-length human p21 cDNA expresses one major polypeptide, amino acid 139 in human p21 seems to play a similar role as that in canine p21 for various migration patterns. The lower bands in the middle row (*lanes 9-16*) were artificially drawn to indicate that the expressed human p21 proteins are corresponding to the upper band of the dog p21 doublets.

Discussion

The Expression Pattern of the p53 Family Members in Canine Cells

The need for more appropriate animal models in cancer research has led researchers to consider companion dogs with spontaneously occurring neoplasms as a valuable and still underused resource. Due to the similarity in genes and physiology between dogs and humans, dogs may act as environmental sentinels, serve as a model to address cancer etiology in humans, and be enrolled in therapeutic trials and thus facilitate clinic applications of potential drugs to humans. Because little is known about the pathogenesis of tumors in companion dogs, we have sought to establish a dog model for the p53 family pathway. We found that canine p53 is up-regulated in response to DNA damage and MDM2 inhibition, which is accompanied by induction of p53 target genes, *p21* and *MDM2*. We also found that p63 and p73 are expressed in canine cells and induced in response to DNA damage but not

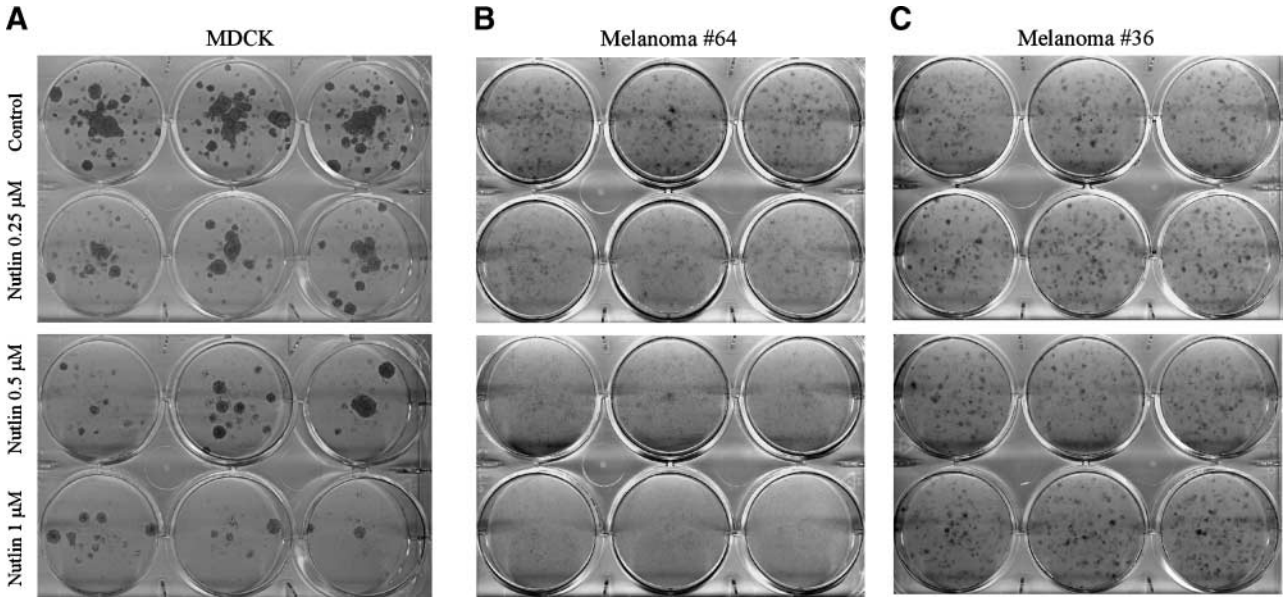

FIGURE 5. Nutlin-3 inhibits the ability of canine melanoma cells to form colony in a p53- and dosage-dependent manner. Cells (2,000 per well) were seeded in a six-well plate for 24 h, followed by treatment with or without nutlin-3 at a concentration of 0.25, 0.5, or 1 μmol/L for 10 d. Cells were then stained with crystal violet for colony formation efficiency.

MDM2 inhibition. This suggests that the canine p53 family members, like their human counterparts, can be up-regulated under a stress condition.

The Role of the p53 Family in Growth Suppression

Both human and dog melanomas are highly resistant to radiation and chemotherapy but low in p53 mutation (22-24). In this study, we found that among the five melanoma cell lines, only one is deficient in p53 whereas the rest of four lines seem to have a functional p53 pathway. Interestingly, the p53-deficient cell line has much higher levels of p63 and p73 compared with the other melanoma cell lines we tested. We also found that the four melanoma cell lines containing wild-type p53 are sensitive to DNA-damaging agents and MDM2 inhibitor nutlin-3. In contrast, the p53-deficient melanoma cell line is insensitive to MDM2 inhibitor but still relatively sensitive to DNA-damaging agents, suggesting that inhibition of cell growth in response to DNA damage is likely due to p63 and p73. Thus, the five melanoma lines are categorized into two groups: one with functional wild-type p53 along with low expression levels of p63 and p73, and the other without functional p53 but with high expression levels of p63 and p73. The finding that nutlin-3 can suppress cell growth in canine melanoma and osteosarcoma cells in a p53- and dosage-dependent manner leads us to hypothesize that dog spontaneous tumor models should be explored to test the feasibility of targeting the p53 pathway to manage certain cancers in humans, considering that the induced nonspontaneous mouse tumors are not ideal for studying spontaneous human tumors.

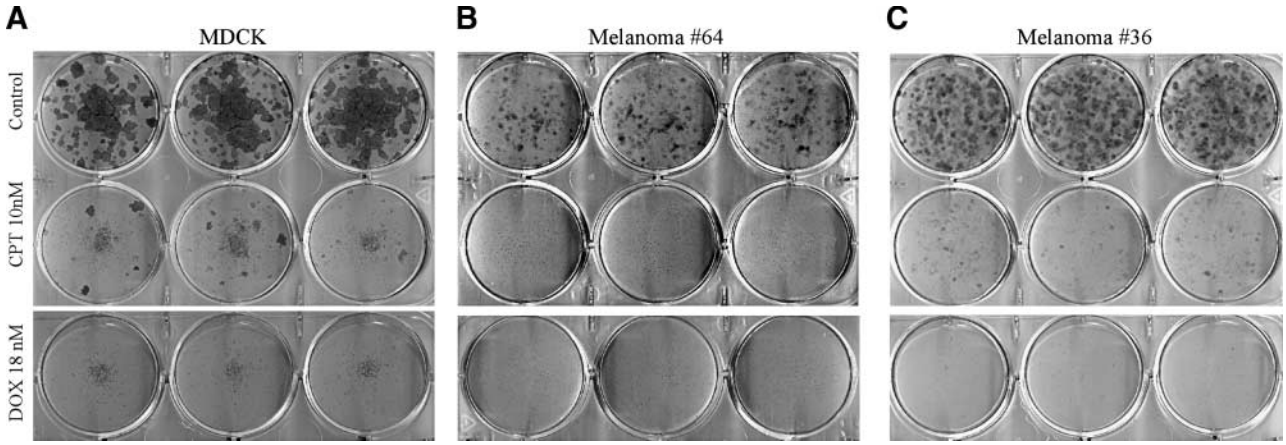

FIGURE 6. The sensitivity of MDCK and melanoma cells to DNA damage agents was measured by colony formation assay. Cells (2,000 per well) were seeded in a six-well plate for 24 h, followed by treatment with or without 10 nmol/L camptothecin or 18 nmol/L doxorubicin for 10 d. Cells were then stained with crystal violet for colony formation efficiency.

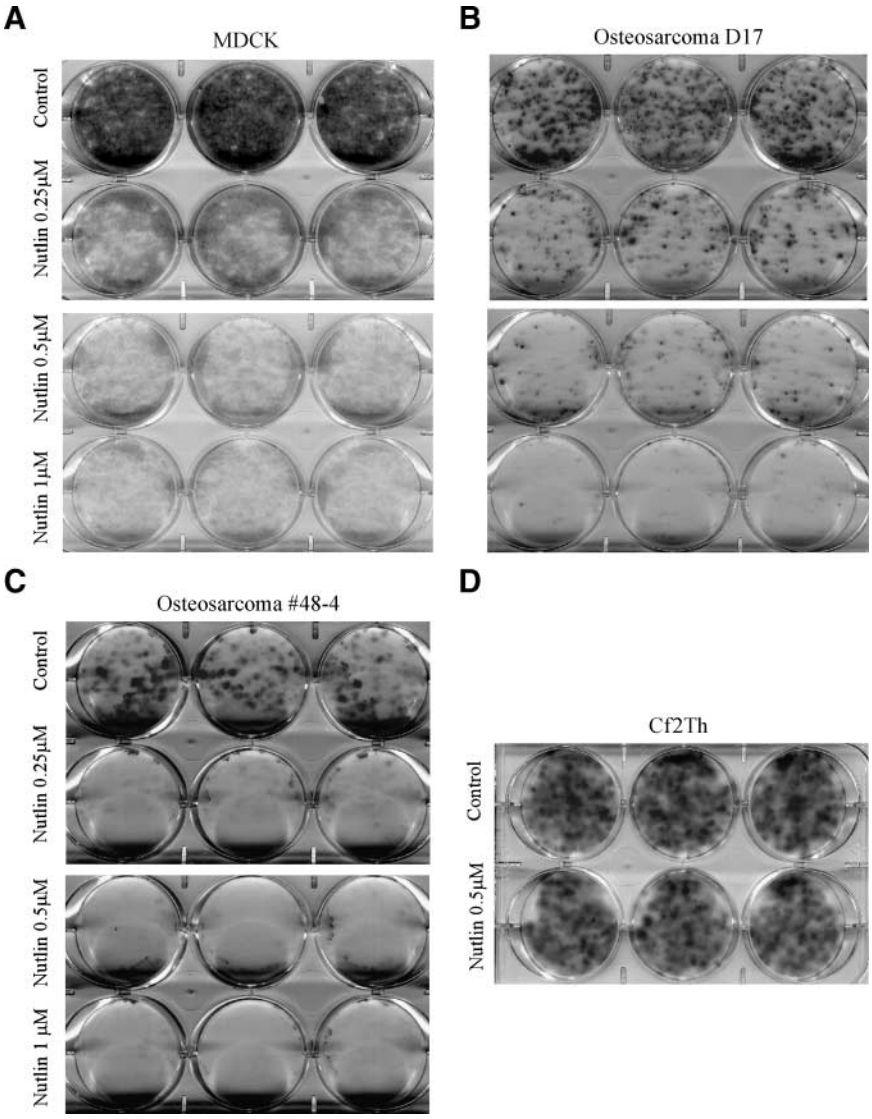

FIGURE 7. Nutlin-3 inhibits the ability of canine osteosarcoma cells, but not Cf2Th cells, to form colony. **A** to **C.** MDCK (**A**) and osteosarcoma (**B** and **C**) cells were seeded at 2,000 per well in a six-well plate for 24 h, followed by treatment with or without nutlin-3 at a concentration of 0.25, 0.5, or 1 μmol/L for 10 d. Cells were then stained with crystal violet for colony formation efficiency. **D.** Cf2Th cells were seeded at 2,000 per well in a six-well plate for 24 h, followed by treatment with or without 0.5 μmol/L nutlin-3 for 10 d.

The Identification of a Novel Isoform of Canine and Possibly Human p21

In this study, we have found that canine p21 is expressed as two isoforms, both of which can be induced in cells on treatment with DNA damage agents or nutlin-3. We then mapped the canine *p21* gene and found that the region of amino acids 129 to 142 is required for the expression of two canine p21 isoforms. Interestingly, only three amino acids (i.e., amino acids 129, 133, and 136) are different between dog and human p21 (Fig. 9). In addition, we showed that amino acid 139 is one of the key determinants for the expression of two p21 isoforms (Fig. 10F). It is possible that these variations are responsible for the increased expression of the small isoform of p21 in the dog. Whereas the full-length human p21 cDNA expresses one major polypeptide, amino acid 139 in human p21 seems to play a similar role as that in canine p21 for various migration patterns (Fig. 10F). Thus, future studies to use a hybrid p21 construct from the human and canine *p21* genes would be likely to uncover the mechanism by which various p21 isoforms are generated and how the migration patterns for various mutated p21 proteins occur. One possibility is that in both normal and cancer cells, trans-splicing of mRNAs has been shown to generate a novel isoform of a gene (25) and might be responsible for the formation of two p21 isoforms in dog.

Several alternate human p21 transcripts have been identified by us and other groups (26-28). However, these human p21 transcripts encode the same protein except p21B (26). Notably, Tchou et al. (29) reported that a fast-migrating COOH-terminal truncated p21 protein was expressed in A549 lung carcinoma cells on treatment with phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, a potent activator of protein kinase C. In addition, Poon et al. (30) found that a p21 isoform, named p21Δ, can be induced in several tumor cells by UV irradiation. Interestingly, these p21 isoforms are found to be expressed in the cytoplasm although it is not certain how these isoforms are generated. Indeed, increased cytosolic p21 expression has been found in breast cancer, which is linked to poorer prognosis (31, 32). These observations point out a

possibility that the human *p21* gene expresses other isoforms, which may have different biochemical and biological functions. We believe that by elucidating how dog p21 is expressed as two isoforms and whether these isoforms have common or distinct functions, we may be able to uncover the mechanism by which various human p21 isoforms are generated and the potential common and distinct functions for these human p21 isoforms.

Materials and Methods

Reagents

Antibodies against p53, p21, MDM2, p63, 53BP1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology. Anti-actin and DAPI were purchased from Sigma. BL906 antibody was purchased from Bethyl Laboratories.

Cell Culture, Clonogenic Assay, and Transfection

Canine melanoma and osteosarcoma cell lines have been defined and used as previously described. MDCK (ATCC #CCL-34), D17 (ATCC #CCL-183), Cf2Th (ATCC #CRL-1430), and HEK293T cell lines were purchased from ATCC. These cells were maintained in DMEM (Dulbecco-Vogt Modified Eagle's MEM) supplemented with 10% fetal bovine serum and 1% nonessential amino acid. Lipofectamine 2000 was used for transfection of p21 cDNAs according to the manufacturer's instructions (Invitrogen). For detection of p53 protein, cells were treated with or without 250 μmol/L camptothecin, 250 ng/mL doxorubincin, or 4 μmol/L nutlin-3 for 12 h. For colony formation assay, 2,000 cells per well in triplicate in a six-well plate were seeded. After 24 h, cells were continuously treated with or without nutlin-3 (0.25, 0.5, or 1.0 μmol/L), 10 nmol/L camptothecin, or 18 nmol/L doxorubicin for 10 d. Cells were then fixed in methanol/glacial acetic acid (7:1), washed with water, and stained with crystal violet (0.2 g/L).

Immunofluorescence Assay

The assay was done as described (33). Briefly, cells were grown on four-well chamber slides and treated as indicated. Cells were then fixed and incubated with primary antibody overnight, followed by incubation with FITC- or Texas red–conjugated secondary antibodies (Jackson ImmunoResearch and Molecular Probes) for 2 h. Cells were also stained with DAPI (Sigma) to visualize nuclei. Intracellular localization of proteins was analyzed by immunofluorescence microscopy.

Western Blot Analysis

The assay was done as described (34). Briefly, cell lysates were made with 2× SDS sample buffer and boiled for 5 min.

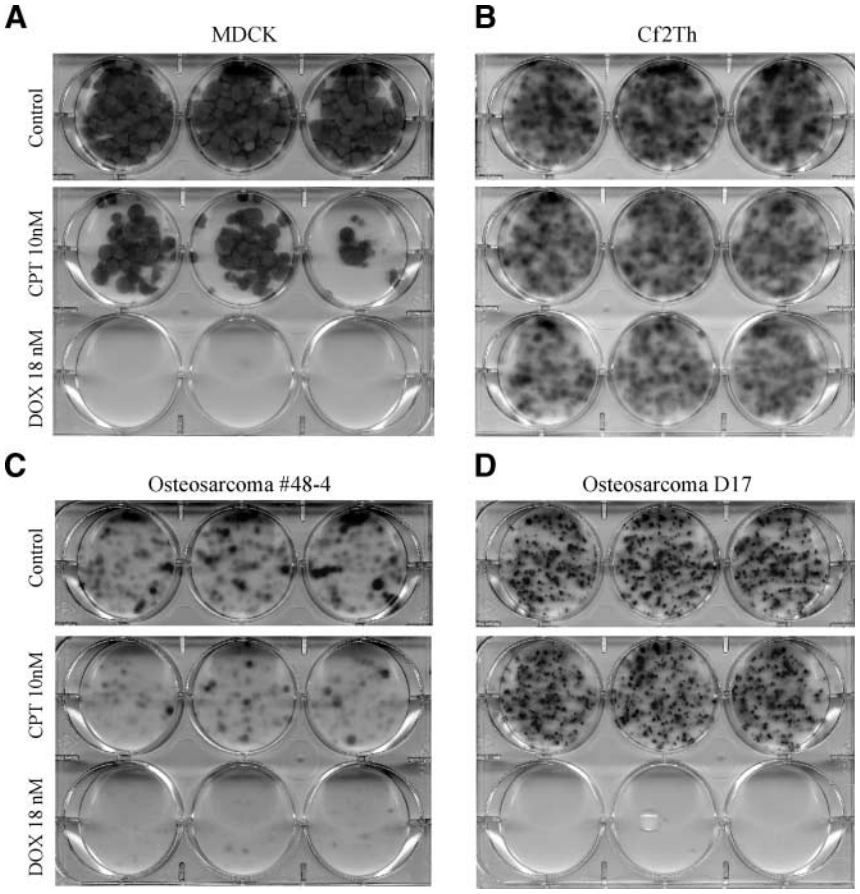

FIGURE 8. The effect of DNA damage on colony formation by MDCK, Cf2Th, and osteosarcoma cell lines. Cells (2,000 per well) were seeded in a six-well plate for 24 h, followed by treatment with or without 10 nmol/L camptothecin or 18 nmol/L doxorubicin for 10 d. Cells were then stained with crystal violet for colony formation efficiency.

Proteins were then resolved in an SDS-PAGE gel and transferred onto nitrocellulose membranes. Membranes were then subjected to blocking, washing, antibody incubation, and detection by enhanced chemiluminescence.

Plasmids

To clone canine p21 cDNA, total RNA was isolated with Trizol reagent according to the manufacturer's instructions (Invitrogen) and reverse transcription-PCR was done with the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The PCR program used for amplification was (*a*) 94°C for 5 min, (*b*) 94°C for 45 s, (*c*) 58°C for 45 s, (*d*) 72°C for 1 min, and (*e*) 72°C for 10 min. From steps *b* to *d*, the cycle was repeated 25 times. The primers used for amplifying full-length p21-coding cDNA were upstream primer 5′-GCCATGTCGGAGCCGTCCAGGG-3′ and downstream primer 5′-ATCCGAATTCAGATTAGGGCTTCCTCTTGGAG-3′. The PCR products were cloned into pGEMT-easy vector for sequencing. To generate canine p21(38-164), a PCR fragment was amplified using the full-length p21-coding cDNA as a template with upstream primer 5′-ATGGCCAGCTGTGTGCAAGA-3 and downstream primer 5′-ATCCGAATTCAGATTAGGGCTTCCTCTTGGAG-3′. The PCR product was then cloned into pcDNA3 vector at *Eco*RI and *Hin*dIII sites. To generate HA-tagged full-length p21-coding cDNA, a PCR fragment was amplified using the full-length p21-coding cDNA as a template with upstream primer 5′-ATCCAAGCTTGCCGCCATGTACCCATACGATGTTCCAGATTACGCTTCGGAGCCGTCCAGGGACG-3′ and downstream primer 5′-ATCCGAATTCAGATTAGGGCTTCCTCTTGGAG-3′. The PCR product was then cloned into pcDNA3 vector at *Eco*RI and *Hin*dIII sites. To generate various HA-tagged p21 mutants, various PCR fragments were amplified using the same upstream primer for HA-tagged full-length p21-coding cDNA and a specific downstream primer for each mutant. These PCR products were then cloned into pcDNA3 vector at *Eco*RI and *Hin*dIII sites to generate each individual canine p21 mutant. The downstream primer for canine p21(1-152) is 5′-ATCCGAATTCTTAGTGATAGAAATCTGTCATGC-3′. The downstream primer for canine p21(1-142) is 5′-ATCCGAATTCTTACCGTTTTCGGCCCTGAGAGG-3′. The downstream primer for canine p21(1-141) is 5′-ATCCGAATTCTTATTTTCGGCCCTGAGAGGTGCC-3′. The downstream primer for canine p21(1-140) is 5′-ATCCGAATTCTTATCGGCCCTGAGAGGTGCCAGGCAC-3′. The downstream primer for canine p21(1-139) is 5′-ATCCGAATTCTTAGCCCTGAGAGGTGCCAGGCAC-3′. The downstream primer for canine p21(1-138) is 5′-ATCCGAATTCTTACTGAGAGGTGCCAGGCACAC-3′. The downstream primer for canine p21(1-136) is 5′-ATCCGAATTCTTAGGTGCCAGGCACACCCGGGGA-3′. The downstream primer for canine p21(1-129) is 5′-ATCCGAATTCTTATGCCTCAGGCCGCTCAGGGG-3′. The downstream primer for canine p21(1-121) is 5′-TTAAGGCAGGAGGGTGCAGGTCAG-3′. The downstream primer for canine p21(1-93) is 5′-TTACTTGCCCCCTCCCAGGTCATC-3′. To generate various human HA-tagged p21 mutants, a similar strategy was used as that for canine p21 mutants except that different primers were used. Specifically, the upstream primer for the full-length human p21 and various p21 mutants is 5′-ATCCAAGCTTGCCGCCATGTACCCATACGATGTTCCAGATTACGCTCAGAACCGGCTGGGGATG-3′. The downstream primer for human p21(1-152) is 5′-ATCCGAATTCTTAGTGGTAGAAATCTGTCATGCTGGTC-3′.

Sequence alignment between Human and Dog p21 proteins
Identities = 136/164 (82%), Positives = 146/164 (89%), Gaps = 0/164 (0%)

Sequence alignment between Human and Mouse p21 proteins
Identities = 125/164 (76%), Positives = 134/164 (81%), Gaps = 5/164 (3%)

```
Dog     1    MSEPSRDALPIPHGSKACRRLFGPVDSEQLRRDCDALMASCVQEARERWNFDFVTETPLE  60
             MSEP+ D    P GSKACRRLFGPVDSEQL RDCDALMA C+QEARERWNFDFVTETPLE
Human   1    MSEPAGDVRQNPCGSKACRRLFGPVDSEQLSRDCDALMAGCIQEARERWNFDFVTETPLE  60
             MS P GDVR  P  SK CR LFGPVDSEQL RDCDALMAGC+QEARERWNFDFVTETPLE
Mouse   1    MSNP-GDVRPVPHRSKVCRCLFGPVDSEQLRRDCDALMAGCLQEARERWNFDFVTETPLE  59

Dog     61   GDFAWERVRGLGLSKVSLPAGPRGGRDDLGGGKRPGTSPALLQGTAQEDHLDLSLTCTLL  120
             GDFAWERVRGLGL K+ LP GPR GRD+LGGG+RPGTSPALLQGTA+EDH+DLSL+CTL+
Human   61   GDFAWERVRGLGLPKLYLPTGPRRGRDELGGGRRPGTSPALLQGTAEEDHVDLSLSCTLV  120
             G+F WERVR LGLPK+YL +   R RD+LGG +RP TS ALLQG A EDHV LSLSCTLV
Mouse   60   GNFVWERVRSLGLPKVYL-SPGSRSRDDLGGDKRPSTSSALLQGPAPEDHVALSLSCTLV  118

Dog     121  PHSPERPEASPGVPGTSQGRKRRQTSMTDFYHSKRRLIFSKRKP  164
             P S E+ E SPG PG SQGRKRRQTSMTDFYHSKRRLIFSKRKP
Human   121  PRSGEQAEGSPGGPGDSQGRKRRQTSMTDFYHSKRRLIFSKRKP  164
                 E+ E SPGGPG SQGRKRRQTS+TDFYHSKRRL+F KRKP
Mouse   119  ---SERPEDSPGGPGTSQGRKRRQTSLTDFYHSKRRLVFCKRKP  159
```

FIGURE 9. Identification of a novel isoform of canine p21. Sequence similarity among dog, human, and mouse p21 proteins.

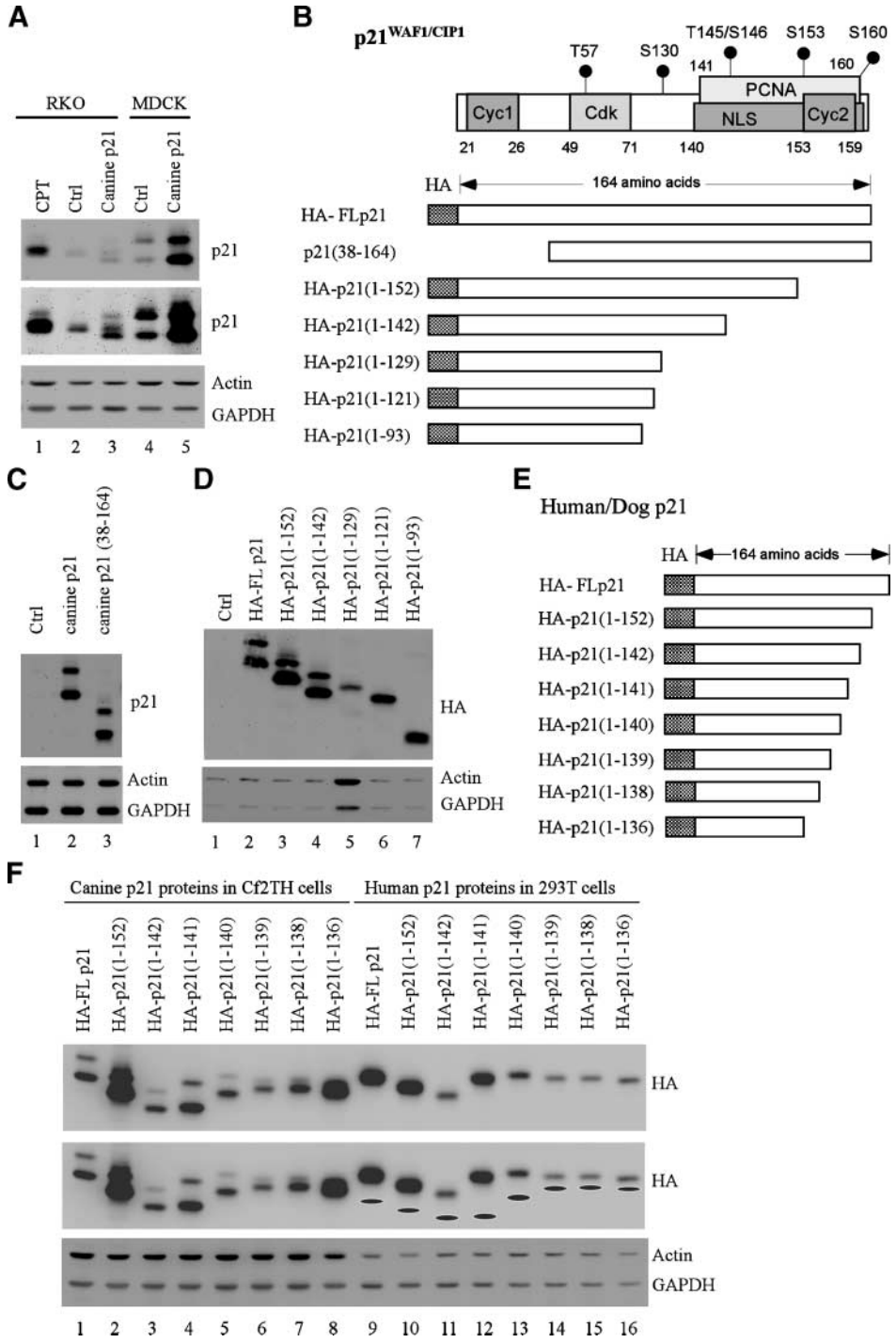

FIGURE 10. **A.** Overexpression of canine p21 in RKO and MDCK cells. Three micrograms of pCDNA3 control vector and canine full-length p21-expressing vector were transfected into MDCK or RKO cells for 24 h, and then the level of canine p21 protein was measured by Western blot analysis with anti-p21 antibody. To stabilize endogenous human p21 in RKO cells as a positive control, RKO cells were treated with 250 nmol/L camptothecin for 12 h. **B.** Schematic presentation of canine p21 protein structure and various canine p21 mutants. **C.** Overexpression of canine mutant p21(38-164) and full-length canine p21 in Cf2Th cells. Three micrograms of control pCDNA3 vector and pCDNA3 vector that expresses canine mutant p21(38-164) or full-length canine p21 were transfected into Cf2Th cells for 24 h, and then the levels of p21 proteins were measured by Western blot analysis with C-19 anti-p21 antibody. **D.** Identification of the region in the p21 protein required for the expression of two isoforms. Three micrograms of control pCDNA3 vector and various canine mutant p21-expressing vectors, as shown in **B**, were transfected into Cf2Th cells for 24 h, and then the levels of p21 proteins were measured by Western blot analysis with anti-HA antibody. **E.** Schematic presentation of wild-type and various mutated canine and human p21 constructs. **F.** A similar migration pattern of p21 proteins between canine and human. Wild-type and various mutated canine p21 constructs were transfected into Cf2TH cells and the levels of the canine p21 proteins were measured with anti-HA antibody (*lanes 1-8*). Similarly, wild-type and various human p21 constructs were transfected into 293T cells and the levels of human p21 proteins were also measured with anti-HA antibody (*lanes 9-16*). The lower bands in the middle row (*lanes 9-16*) were artificially drawn to indicate that the expressed human p21 proteins are corresponding to the upper band of the dog p21 doublets.

The downstream primer for human p21(1-142) is 5′-ATCCGAATTCTTACCGTTTTCGACCCTGAGAGTCTCC-3′. The downstream primer for human p21(1-141) is 5′-ATCCGAATTCTTATTTTCGACCCTGAGAGTCTCCAGG-3′. The downstream primer for human p21(1-140) is 5′-ATCCGAATTCTTATCGACCCTGAGAGTCTCCAGG-3′. The downstream primer for human p21(1-139) is 5′-ATCCGAATTCTTAACCCTGAGAGTCTCCAGGTCCACC-3′. The downstream primer for human p21(1-138) is 5′-ATCCGAATTCTTACTGAGAGTCTCCAGGTCCACC-3′. The downstream primer for human p21(1-136) is 5′-ATCCGAATTCTTAGTCTCCAGGTCCACCTGGGGAC-3′.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Jemima Hall for administrative assistance.

References

1. Ko LJ, Prives C. p53: puzzle and paradigm. Genes Dev 1996;10:1054–72.

2. Yang A, Kaghad M, Wang Y, et al. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 1998;2:305–16.

3. Kaghad M, Bonnet H, Yang A, et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 1997;90:809–19.

4. Donehower LA, Harvey M, Slagle BL, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 1992; 356:215–21.

5. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 1999;398: 708–13.

6. Yang A, Schweitzer R, Sun D, et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 1999;398: 714–8.

7. Yang A, Walker N, Bronson R, et al. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 2000;404:99–103.

8. Levine RA, Fleischli MA. Inactivation of p53 and retinoblastoma family pathways in canine osteosarcoma cell lines. Vet Pathol 2000;37:54–61.

9. Rungsipipat A, Tateyama S, Yamaguchi R, Uchida K, Miyoshi N, Hayashi T. Immunohistochemical analysis of c-yes and c-erbB-2 oncogene products and p53 tumor suppressor protein in canine mammary tumors. J Vet Med Sci 1999; 61:27–32.

10. Ginn PE, Fox LE, Brower JC, Gaskin A, Kurzman ID, Kubilis PS. Immunohistochemical detection of p53 tumor-suppressor protein is a poor indicator of prognosis for canine cutaneous mast cell tumors. Vet Pathol 2000;37: 33–9.

11. Gordon IK, Ye F, Kent MS. Evaluation of the mammalian target of rapamycin pathway and the effect of rapamycin on target expression and cellular proliferation in osteosarcoma cells from dogs. Am J Vet Res 2008;69: 1079–84.

12. Chen SC, Chen CH, Chern CL, et al. *p*-Phenylenediamine induces p53-mediated apoptosis in Mardin-Darby canine kidney cells. Toxicol In Vitro 2006; 20:801–7.

13. Nelson WG, Kastan MB. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol 1994; 14:1815–23.

14. Vassilev LT, Vu BT, Graves B, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004;303:844–8.

15. el-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817–25.

16. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature 1997;387:296–9.

17. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature 1997;387:299–303.

18. Rappold I, Iwabuchi K, Date T, Chen J. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. J Cell Biol 2001;153:613–20.

19. Anderson L, Henderson C, Adachi Y. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. Mol Cell Biol 2001;21:1719–29.

20. Petitjean A, Cavard C, Shi H, Tribollet V, Hainaut P, Caron de Fromentel C. The expression of TA and ΔNp63 are regulated by different mechanisms in liver cells. Oncogene 2005;24:512–9.

21. Harms K, Nozell S, Chen X. The common and distinct target genes of the p53 family transcription factors. Cell Mol Life Sci 2004;61:822–42.

22. Volkenandt M, Schlegel U, Nanus DM, Albino AP. Mutational analysis of the human p53 gene in malignant melanoma. Pigment Cell Res 1991;4:35–40.

23. Montano X, Shamsher M, Whitehead P, Dawson K, Newton J. Analysis of p53 in human cutaneous melanoma cell lines. Oncogene 1994;9:1455–9.

24. Roels S, Tilmant K, Ducatelle R. p53 expression and apoptosis in melanomas of dogs and cats. Res Vet Sci 2001;70:19–25.

25. Li H, Wang J, Mor G, Sklar J. A neoplastic gene fusion mimics trans-splicing of RNAs in normal human cells. Science 2008;321:1357–61.

26. Nozell S, Chen X. p21B, a variant of p21(Waf1/Cip1), is induced by the p53 family. Oncogene 2002;21:1285–94.

27. Gartel AL, Radhakrishnan SK, Serfas MS, Kwon YH, Tyner AL. A novel p21WAF1/CIP1 transcript is highly dependent on p53 for its basal expression in mouse tissues. Oncogene 2004;23:8154–7.

28. Radhakrishnan SK, Gierut J, Gartel AL. Multiple alternate p21 transcripts are regulated by p53 in human cells. Oncogene 2006;25:1812–5.

29. Tchou WW, Rom WN, Tchou-Wong KM. Novel form of p21(WAF1/CIP1/SDI1) protein in phorbol ester-induced G_2/M arrest. J Biol Chem 1996;271: 29556–60.

30. Poon RY, Hunter T. Expression of a novel form of p21Cip1/Waf1 in UV-irradiated and transformed cells. Oncogene 1998;16:1333–43.

31. Wong NA, Mayer NJ, Anderson CE, et al. Cyclin D1 and p21 in ulcerative colitis-related inflammation and epithelial neoplasia: a study of aberrant expression and underlying mechanisms. Hum Pathol 2003;34:580–8.

32. Yang W, Klos KS, Zhou X, et al. ErbB2 overexpression in human breast carcinoma is correlated with p21Cip1 up-regulation and tyrosine-15 hyperphosphorylation of p34Cdc2: poor responsiveness to chemotherapy with cyclophosphamide methotrexate, and 5-fluorouracil is associated with Erb2 overexpression and with p21Cip1 overexpression. Cancer 2003;98: 1123–30.

33. Jung EJ, Liu G, Zhou W, Chen X. Myosin VI is a mediator of the p53-dependent cell survival pathway. Mol Cell Biol 2006;26:2175–86.

34. Chen X, Bargonetti J, Prives C. p53, through p21 (WAF1/CIP1), induces cyclin D1 synthesis. Cancer Res 1995;55:4257–63.