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# Phenotype-Rescue of Cyclin-Dependent Kinase Inhibitor p16/INK4A Defects in a Spontaneous Canine Cell Model of Breast Cancer

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## ABSTRACT

Mammary cancer is among the most frequently observed canine tumors in unspayed female dogs resulting in death due to metastatic disease. These tumors are excellent models of human breast cancer but until recently there was only anecdotal evidence regarding underlying genetic defects. We recently reported expression defects in the cyclin-dependent kinase p21/Cip1 and p53 among three independent canine mammary tumor (CMT) cell lines derived from spontaneous canine mammary cancers. We investigated further defects in the same three cell lines focusing on additional tumor suppressor gene defects in cyclin-dependent kinase inhibitors. p27/KIP1 appeared normally expressed and did not appear to encode inactivating mutations. In contrast, expression of p16/INK4A was defective/absent in two cell lines and normal/slightly induced in the third cell line. To determine if defects were causative in maintaining the transformed phenotype, a p16/INK4A transgene was permanently transfected followed by selection and single cell cloning. CMT/p16 clones were characterized for transgene expression, p16 protein content and phenotype including proliferation rate, cell cycle phase distribution, contact inhibition, substrate dependent cell growth and cell morphology. All cell lines appeared unique yet clear indications of phenotype rescue due to p16/INK4A transgene complementation were observed suggesting that defects in p16 expression were present in all three. In some cases cellular senescence also appeared to be induced. These data provide evidence supporting p16/INK4A mutations as causative defects promoting transformation in canine mammary cancer and further characterizes tumor suppressor gene defects with functional consequences in these cells supporting their application as spontaneous animal models of human disease. J. Cell. Biochem. 106: 491–505, 2009. 

**KEY WORDS:** CKI; CELL CYCLE; CDK; MAMMARY CANCER; CANINE

Cyclin-dependent kinase (CDKs)/cyclin complexes compose the enzymatic core of the principle regulatory complexes that govern the transition checkpoints during sequential phases of each cell cycle [Forsburg and Nurse, 1991; Bird, 1997, 2003; Ekholm and Reed, 2000]. CDK activity is controlled at multiple levels including transcription and degradation of its cyclin cofactor, post-translational modification of both cyclin and CDK and through association with a complex family of cyclin-dependent kinase inhibitors (CKIs) to form CDK integration complexes [Pavletich, 1999; Smits et al., 2000; Frey et al., 2001]. CKIs are grouped in two classes: the Cip/KIP family including p21/Cip1, p27/KIP1 and p57/KIP2 and, secondly, the INK4 family including p16/INK4A, p15/INK4B, p18/INK4C, and p19/INK4D [Ekholm and Reed, 2000; Bostrom et al., 2001; Christopher et al., 2002; Tyner and Gartel, 2003]. Each CKI appears to be selective for specific cyclin/CDK complexes and can even be cell type and stimulus dependent. Activation of cyclin/CDK requires relief from inhibition by bound CKIs of which most have been shown to be tumor suppressor genes through loss of their ability to suppress proliferation [Ekholm and Reed, 2000; Tyner and Gartel, 2003]. Single CKI knockout mice have a variable and generally non-severe phenotype reflecting probable redundancy in function among these genes [Tyner and Gartel, 2003]. p57 knockout mice show the most obvious and lethal defects from a single CKI gene deletion while other CKI knockouts result in far more subtle defects and changes [reviewed in Nakayama and Nakayama, 1998].

p27/KIP1 is the CKI most frequently associated with cell cycle arrest and exit from the cell cycle [Sherr and Roberts, 1999]. Levels of p27/KIP1 rise as cells exit the cell cycle and, conversely, drop due to ubiquitination and proteolysis, as cells re-enter the cell cycle and may even be inactivated by direct cyclin/CDK sequestering [Malek et al., 2001; Cheng and Scadden, 2002]. Evidence has been reported that Cip/KIP CKIs may be differentially active in different cell types as p21, p27, and p57 appear to be expressed in different tissues during mouse embryo development and differentially in adult

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tissues [Nakayama and Nakayama, 1998; Cheng and Scadden, 2002]. Counterintuitively, an activation of cyclin/CDKs has also been described for Cip/KIP inhibitors through their chaperonin-like activity that may promote active complex assembly [LaBaer et al., 1997].

The INK4 CKI family includes p16/INK4A which primarily associates with cyclin D/CDK4 and cyclin D/CDK6 complexes [Tyner and Gartel, 2003]. INK4 CKIs bind cyclin/CDK complexes and appear dominant over other activating factors until they are inactivated [Pavletich, 1999]. Redundancy also appears to be built into this CKI function. Although p27 appears to be the principle regulator of G0/G1 phase transition, p16/INK4A, an alternative open reading frame transcript from the p16/INK4A locus, p19ARF, and p15 INK4B also appear to function in G0/G1 phase maintaining proliferative senescence [Carnero et al., 2000; Ekholm and Reed, 2000; Malumbres et al., 2000; Sandhu et al., 2000; Wei et al., 2001]. INK4 binding may destabilize cyclin/CDK complexes preventing assembly during G1 phase and p16/INK4A and p21/Cip1 accumulation has been demonstrated in senescent cells implicating them in transition out of the cell cycle and in regulating differentiation [Nakayama and Nakayama, 1998; Jeffrey et al., 2000]. For example, the transcription factor MyoD, that promotes skeletal muscle differentiation and terminal differentiation, activates p21/Cip1 expression ensuring cell cycle arrest during terminal differentiation [Arellano and Moreno, 1997]. Conversely, enhanced p21/Cip1 expression during muscle differentiation can enhance muscle gene expression [Nakayama and Nakayama, 1998]. Redundancy in CKI function is also evident here as p21 knockout mice do not express defective muscle development although p21/p57 double knockout mice do [Zhang et al., 1999]. Thus, the cyclin/CDK integration complexes are required to regulate cell cycle progression and are regulated themselves by a changing array of CKIs providing a subtle interplay between activities that balance cell proliferation, arrest and differentiation.

Canine mammary cancers, particularly the carcinomas, are among the most frequently observed spontaneous neoplasms in dogs [Sartin et al., 1993; Ahern et al., 1996; MacEwen and Withrow, 1996]. The apparent etiology, hormone and age dependence, the aggressive behavior of this tumor and its capacity to metastasize provide a high level of analogy to human breast cancer [Ritt et al., 2000; Koenig et al., 2002; DeInnocentes et al., 2006; Migone et al., 2006]. We have investigated a range of genetic abnormalities present in canine mammary carcinoma in an effort to develop it as a therapeutic model. We have previously identified defects in expression and/or sequence in several genes involved in regulation of cell proliferation and life-span including c-*erb*B-2 (HER2/*neu*), p21/Cip1, p53 and SIRT2 reflecting the similarity of genetic defects observed in this disease in humans and dogs [Ahern et al., 1996; DeInnocentes et al., 2006]. Our previous efforts have also explored the mechanisms regulating CDK1 expression in canine mammary cancer cells [Wolfe et al., 1986; You and Bird, 1995; Liu and Bird, 1998; Ritt et al., 2000; Bird and DeInnocentes, 2004; DeInnocentes et al., 2006; Migone et al., 2006].

Members of the INK4 and Cip/KIP families are conserved in nucleotide sequence between mammalian species in the few cases where this has been studied including regions of very high homology [Ruas and Peters, 1998; Sharpless and DePinho, 1999]. We have used this approach to amplify and clone CKI mRNA sequences from canine mammary tumor (CMT) cells in vitro. We report sequence and expression of three of the most important canine CKI mRNAs involved in cell cycle progression in canine mammary cancer and rescue of the transformed phenotype of these cells by p16/INK4A complementation in *trans*.

## MATERIALS AND METHODS

### CELL CULTURE

CMT12, CMT27, and CMT28 cell lines were obtained from Dr. L. Wolfe and grown in complete L-15 media [Wolfe et al., 1986; DeInnocentes et al., 2006; Migone et al., 2006]. Human epitheliod cervical carcinoma HeLa S3 cells, as well as human breast cancer cells HB131 and MCF7 were also acquired (ATCC). These cell lines and CMT12, CMT27, CMT28 and normal canine fibroblasts (NCFs) were cultured in α-modified Eagle medium (Gibco) with antibiotics (Sigma) and 10% fetal bovine serum (Hyclone) as described [DeInnocentes et al., 2006]. Cells were grown at 100% humidity at 37°C with 5% $CO_2$.

### TRANSFECTION, SELECTION AND SINGLE CELL CLONING

The pBK-RSV/P16 expression clone was obtained from Dr. J. Modiano [Koenig et al., 2002]. Plasmid DNAs in *E. coli* were grown and purified using a Qiagen endotoxin free kit and the DNA was linearized and quantified. Cells were transfected using Effectine transfection reagent (Qiagen) and 1 μg linear plasmid DNA/25 $cm^2$ flask. Conditions were optimized and clones selected in growth media containing G418/Geneticin (100 μg/ml, Gibco) to screen for transfectants. Each cell line was single-cell-sorted into 96-well plates (Corning) on a MoFlo flow cytometer and cell sorter equipped with a cyclone robotic arm plate (Beckman Coulter). Each single cell clone was grown and RNA extracted for analysis in rt-PCR.

### CELL PROLIFERATION AND MICROSCOPY

Cultures of parental CMT cell lines, and transfected clonal lines derived from them, were seeded into 75 $cm^2$ flasks (Corning) to produce an approximate confluency of 20% in growth medium. Cultures were grown to confluency and then allowed to age naturally with regular changes of growth medium. Cells were counted in a hemocytometer without dilution every day for 4 days taking care that cultures did not reach confluence. Cultures were liberated with trypsin digestion, washed and resuspended in complete growth medium and placed on ice prior to counting. Each culture was counted twice in two separate loadings and at least 300 cells were counted for each loading. Each experiment was repeated at least three times. Pictures of live cultures using phase contrast microscopy were achieved with an Olympus CK2 phase contrast inverted microscope and a Nikon Digital Sight digital camera and detector attachment using 10 and 40 times objectives.

### SUBSTRATE-DEPENDENT PROLIFERATION ON SOFT-AGAR

Substrate-dependent growth of individual transfected cell clones and parental cell lines was evaluated in proliferation assays on soft agar [Hamburger and Salmon, 1977]. Wells were prepared in 6-well culture plates (Corning) containing 1.3 ml of solution 1 containing

4% (w/v) Type I Agarose (Fisher) in complete L-15 medium (Gibco) at 37°C. CMT cells were collected by trypsinization, resuspended in 2 ml of L-15 medium, counted and diluted to $1 \times 10^5$/ml (Stock A) and $0.5 \times 10^5$/ml (Stock B) in complete L-15 medium. Then, 0.9 ml of 4% (w/v) of molten agarose (Sea plaque, FMC) solution (solution B) was added to each 7.2 ml of L-15 medium and all solutions were maintained at 37°C. Then, 2.7 ml of the agarose solution was combined with 0.3 ml solution of Stock A, mixed and poured into two wells (1 ml/well) and the process was repeated with Stock B. Positive control NCF cells were also evaluated at $1 \times 10^5$ cells/ml. Lastly, 1 ml of solution B without cells was poured into the last well of each 6 well plate, as a negative control. Plates were incubated at 4°C for 5 min and then at 37°C in a 5% $CO_2$ incubator. Colonies were counted on the 3rd, 4th, and 5th week after the cells were plated. Cells were fed every week (0.5 ml growth medium/well) with solution B. Each experiment was repeated three times.

### FLOW CYTOMETRY AND CELL CYCLE PHASE ANALYSIS

Analysis of cell cycle phase by flow cytometry of propidium iodide labeled DNA was performed on rapidly growing cultures of subconfluent cells (less than 70%) after fixation in ethanol as previously described [You and Bird, 1995; DeInnocentes et al., 2006] (28,30). All flow cytometry was performed on a MoFlo flow cytometer and cell sorter (Beckman Coulter) and cell cycle phase distribution calculated using Summit 4.3 software (Beckman Coulter). Mean percent of cells in each cell cycle phase was calculated as the mean of two to four independent determinations (minimum of $1 \times 10^4$ cells/determination). Standard deviations for CMT cell cycle phase determinations were +/− 0.4 to 2.4.

### PREPARATION OF RNA, PRIMER DESIGN, rt-PCR AND DNA SEQUENCING

Total RNA was extracted from cells using RNA Stat 60 (Tel-Test, Inc.). RNA pellets were resuspended in diethylpyrocarbonate-treated water and stored at −80°C. The concentration of the RNA was determined by absorbance at 260 nm [You and Bird, 1995]. Specific primers (Omega Biotek) were designed to anneal only to the specific sequences and to prime synthesis of cDNA from conserved sequences in dog p16/INK4A (Genbank U92435), p21/Cip1 (Genbank AF076469), and p27/KIP1 (Genbank AY004255) mRNAs. Gene expression was analyzed using rt-PCR of mRNA made from cultured cells in semi-quantitative assays at limiting template dilution and employing a minimum number of rounds of amplification [DeInnocentes et al., 2006]. The primer pair for canine p16/INK4A was previously described (sense 5′-AGCTGCTGCTGCTC-CACGG-3′, antisense 5′-ACCAGCGTGTCCAGGAAGCC-3′) as was the primer pair for canine p21/Cip1 (sense 5′-GACTGTGATGCGC-TAATGGC-3′, antisense 5′-GGGTACAAGACAGTGACAGG-3′, 26). The primer pair for canine p27/KIP1 was designed using DNAStar software (sense 5′-TCTCCTGCGCCGGCACCT-3′, antisense 5′-CTGGAGCGGATGGACGCCA-3′). Each amplicon was gel purified and cloned into vector pCR2.1 (Invitrogen) and sequenced (Auburn University Genomics and Sequencing Laboratory). Sequences of multiple clones for each cDNA were compared with p16/INK4A, p21/Cip1 and p27/KIP1 cDNA sequences from several different species in Genbank as noted.

### WESTERN BLOT

Western blots were performed as described with the following exceptions [You and Bird, 1995]. The blots were blocked for 2 h. The primary antibody used was anti-p16 rabbit polyclonal IgG at a dilution of 1:200 (H-156, Santa Cruz). The secondary antibody used was ImmunoPure goat anti-rabbit IgG (H + L), which was alkaline phosphatase conjugated, at a dilution of 1:1,000 (Pierce/Thermo). The p16 protein bands were photographed and compared with prestained standards (Bio-Rad) to estimate relative size.

## RESULTS

### EXPRESSION AND SEQUENCE ANALYSIS OF CANINE CKIs IN CMT CELLS

RNA populations were isolated from three independent CMT lines (CMT12, CMT27, and CMT28) originally derived from spontaneously occurring canine mammary cancers [Wolfe et al., 1986; You and Bird, 1995]. rt-PCR assays were designed and optimized based on previously published assays or sequences in Genbank. RNAs were amplified and analyzed along with RNAs from HeLa cells, an epithelioid cervical carcinoma, and NCF cells to compare levels of expression of CKI transcripts encoding canine p16/INK4A and p27/KIP1 and as was previously reported for p21/Cip1 [You and Bird, 1995; Bird and DeInnocentes, 2004; DeInnocentes et al., 2006]. Although p27/KIP1 was expressed at comparable and abundant levels in all CMT cell lines, human breast cancer lines and HeLa cells, the levels of expression of p16/INK4A were not comparable (Fig. 1). p16/INK4A was not expressed by CMT12 or CMT27 cells although a

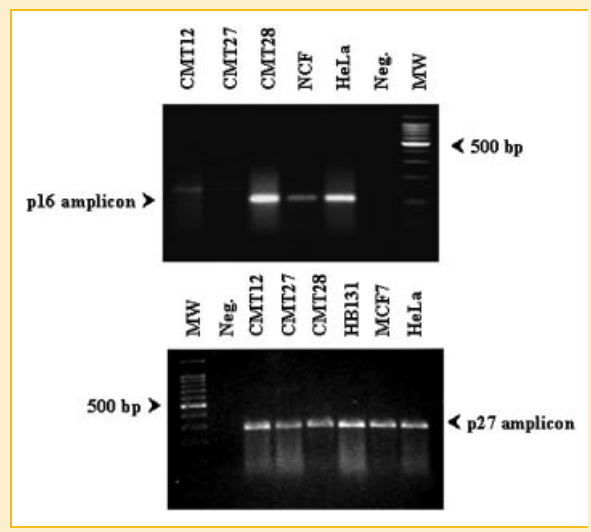


Fig. 1. rt-PCR of CKI tumor suppressor genes in CMT cells. rt-PCR of canine CKI mRNA levels encoding p16/INK4A and p27/KIP1 were evaluated by semi-quantitative rt-PCR in CMT cell lines as well as NCFs and human tumor derived cell lines including HeLa (cervical epithelioid carcinoma) cells and human breast cancer derived cells (MCF7 and HB131). Transcripts were reverse transcribed and amplified as noted and analyzed by agarose gel electrophoresis. Specific amplicons are indicated as is the 500 bp marker (arrows) in the 100 bp marker lane (MW).

TABLE I. Summary of CMT Cell Line Gene Defects*

| | p16/INK4A | p21/Cip1[a] | p27/KIP1 | c-*erb*B-2[b] | p53[a] |
|---|---|---|---|---|---|
| CMT12 | – | + | + | ++ | + |
| CMT27 | – | + | + | ++ | ++ |
| CMT28 | ++ | – | + | +++ | – |

[a]Data from DeInnocentes et al. [2006].
[b]Data from Ahern et al. [1996].
*No expression (–), normal expression (+), overexpression >1.5-fold (++), overexpression >5-fold (+++).

trace amplicon of an unrelated and higher molecular weight was apparent in some CMT12 amplifications (confirmed by DNA sequencing not to be p16-related—data not shown). The levels of expression were also several fold higher in HeLa and CMT28 cells when compared to NCFs. This was contrasted with previously published p21/Cip1 and p53 expression which was similar in all cell lines evaluated except CMT28 where little or no expression was detected [DeInnocentes et al., 2006]. Thus, CMT12 and CMRT27 cells appeared defective in p16/INK4A expression and CMT28 cells appeared defective in p21/Cip1 and p53 expression while overexpressing p16/INK4A. Because p53 varied somewhat between CMT12 and CMT27 cells, these three cell lines provide three unique phenotypes regarding expression of these tumor suppressor genes (Table I).

Amplicons derived from all three canine CKI open reading frames were cloned and multiple clones sequenced revealing unique differences from published examples where those were available (Fig. 2). Alignments of canine p16/INK4A open reading frames (152 bp of 471 bp total) revealed six silent nucleotide changes between the published canine and human sequences (human—Genbank No. L27211 and canine Genbank No. AF234176, 26) and all six of these differences were shared with the NCF and CMT28 p16/INK4A sequences (the only CMT line expressing p16/INK4A) recovered (Fig. 2A). However, both the NCF and CMT28 p16/INK4A sequences also encoded seven additional nucleotide differences, not shared with either published human or canine sequences, including 3

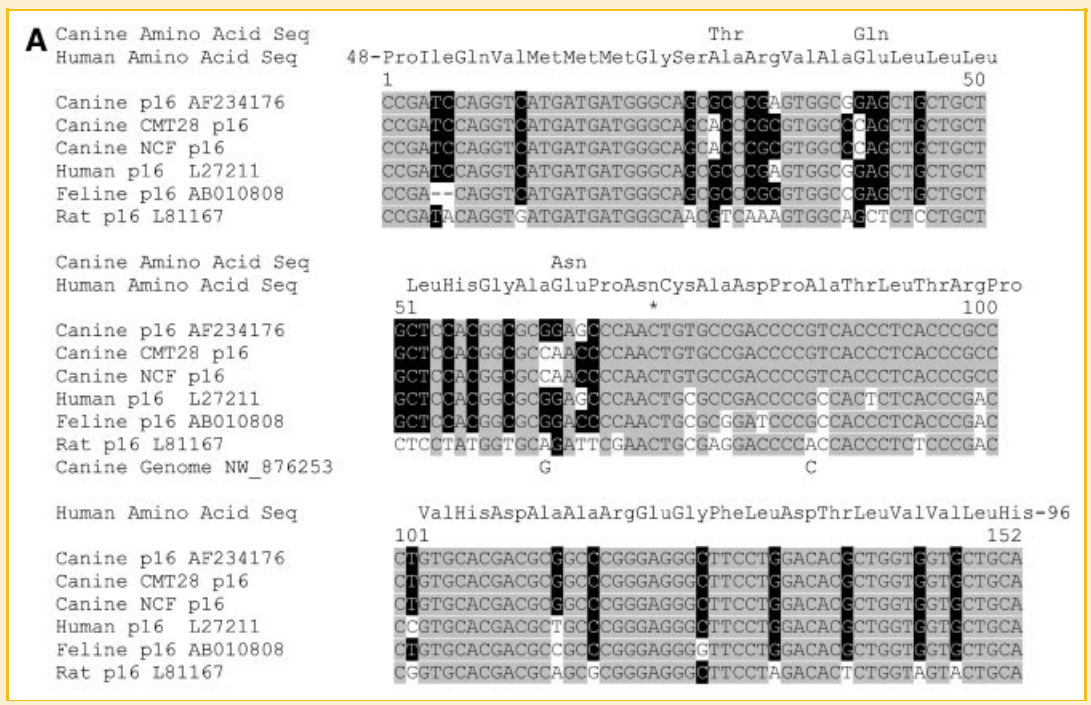


Fig. 2A. Canine and mammalian CKI sequence alignments. DNA sequences representing the transcript cDNAs derived from CMT CKIs were aligned using AlignX (Vector NTI) software and compared to other mammalian CKI sequences of the same regions. A: Canine p16/INK4A homologous amplicons from cell lines CMT28 and NCF (Genbank No. FJ542308 and FJ542309) of 152 bp and representing nucleotides 182–333 of the p16/INK4A ORF encoding amino acids 48–98, with respect to the human sequence, were cloned, sequenced and aligned to p16/INK4A cDNA sequences from rat, domestic cat, human and domestic dog (Genbank accession numbers for p16/INK4A sequences from dog—AF234176, human—L27211, cat—AB010808, and rat—L81167). Differences compared to the p16/INK4A coding region of the canine genome are also noted. Canine amino acid differences noted are extrapolated from NCF and CMT derived sequences as no amino acid differences were noted between the published sequences for canine and human p16/INK4A. B: Canine p21/Cip1 homologous amplicons from cell lines CMT12 (Genbank No. FJ542310) of 359 bp and representing nucleotides 103–461 of p21/Cip1 encoding amino acids 35–154, with respect to the human sequence, were cloned, sequenced and aligned to p21/Cip1 sequences from mouse, domestic cat, human, chimpanzee and domestic dog (Genbank accession numbers for p21/Cip1 sequences from dog—AF076469, human—BT006719, cat—D84650, chimpanzee—XM_518437 and mouse—BC002043). C: Canine p27/KIP1 homologous amplicons from cell line CMT12 (Genbank No. FJ542311) of 295 bp and representing nucleotides 37–329 of the p27/KIP1 ORF encoding amino acids 13–110, with respect to the human sequence, were cloned, sequenced and aligned to p27/KIP1 sequences from mouse, rat, domestic cat, human, domestic pig, domestic chicken and domestic dog (Genbank accession numbers for p27/KIP1 sequences from dog—AY004255, human—NM_004064, cat—D84649, pig—AB083336, chicken—AY094490, mouse—MMU10440, and rat—D83792). Nucleotides conserved across all sequences are noted (black nucleotides on gray) as are divergent nucleotides (black nucleotides on white). For all alignments, nucleotides representing the consensus sequence at locations where these interspecies or inter-individual/allelic base differences were identified are also noted (white nucleotides on black). Amino acid substitutions/differences in individual canine CMT or NCF cell line CKIs are compared above the complete human amino acid sequence extrapolated from respective cDNAs.

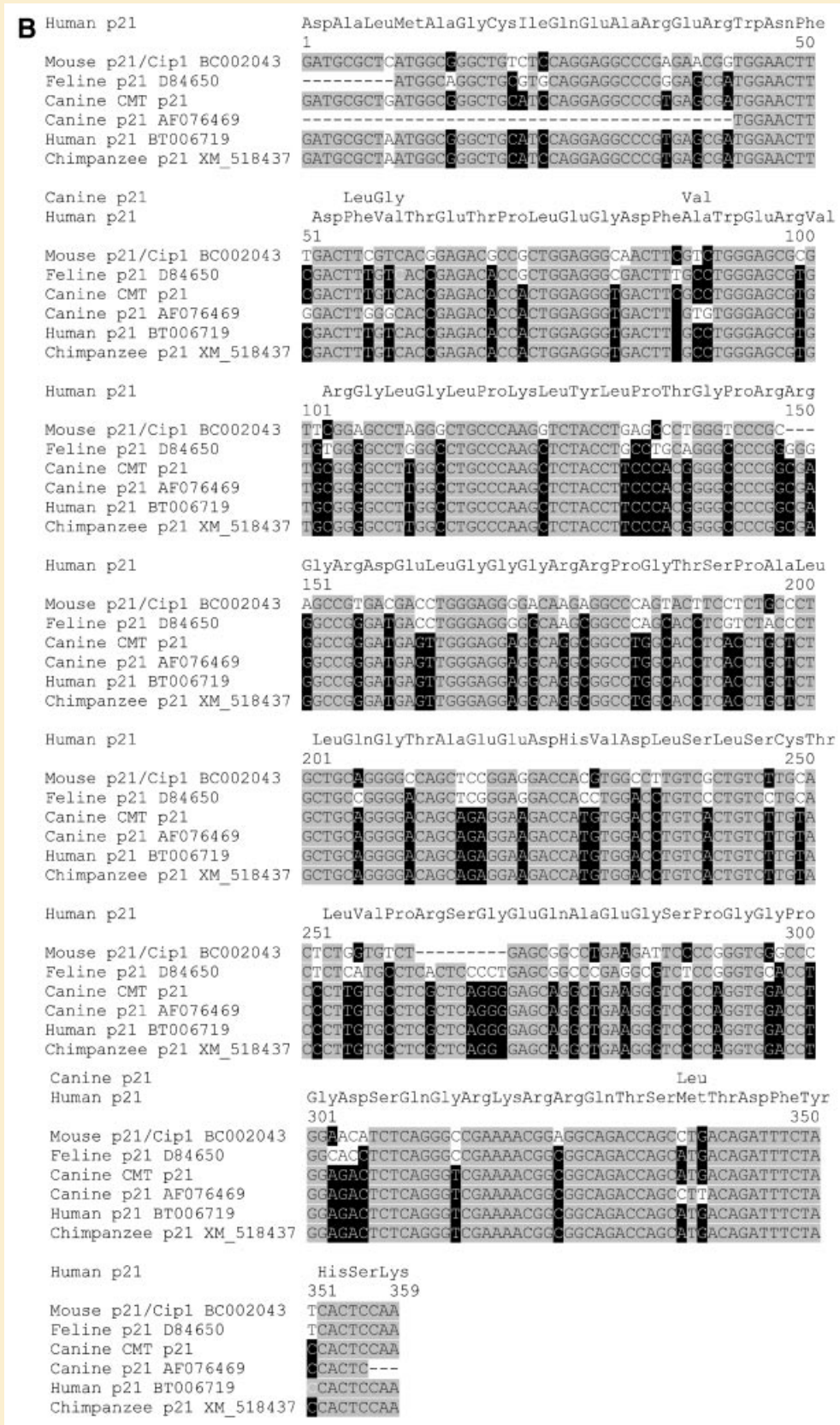


Fig. 2B. (*Continued*)

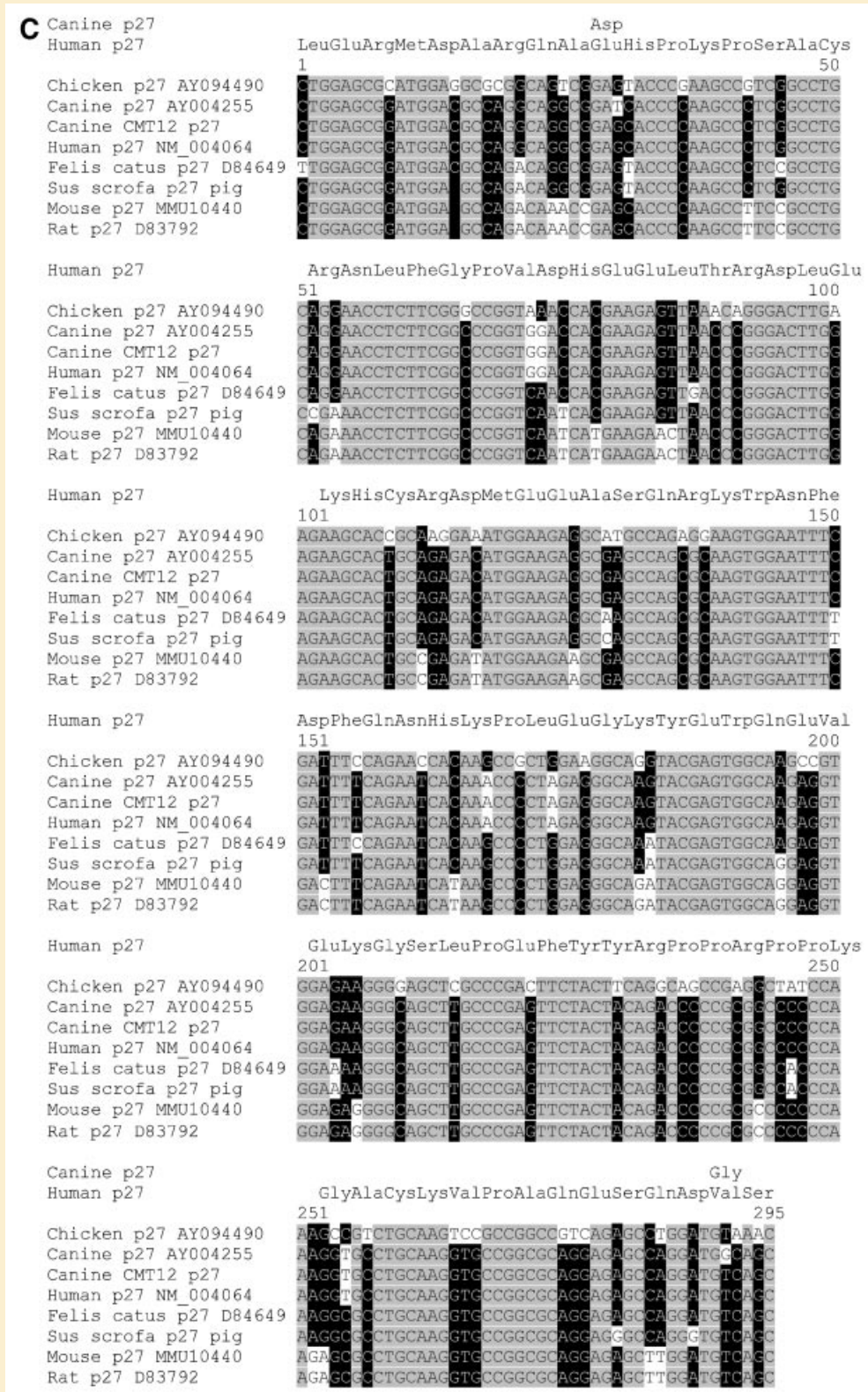


Fig. 2C. (*Continued*)

silent third base differences (nt 33, 39, and 63) and 4 first and third base differences that altered the amino acid encoded in these new canine sequences (nt 28, 40, and 64/66). All three amino acid differences were non-conservative (Glu to Asn and Glu to Gln representing acidic to polar amino acid changes at amino acids 69 and 61, respectively, and an Ala to Thr non-polar to polar amino acid change at amino acid 57) and shared by NCF and CMT28 cells though they were not present in the Genbank sequence for canine p16/INK4A mRNA or the sequence in the canine genome. Thus, canine p16/INK4A sequences recovered from NCF and CMT28 cells encoded ample evidence of expressed non-conserved sequence diversity in 152 nucleotides of the open reading frame (corresponding to human p16/INK4A nt 182–333) common to at least two unrelated dogs of different breeds (pure bred beagle and mixed breed dog, respectively). The three amino acid differences corresponded to less conserved amino acids in the second ankyrin repeat and are not among those residues identified previously as mutated in cancer specimens [Russo et al., 1998].

Canine p21/Cip1 amplicons of 359 bp, representing nt 103–461 and encoding amino acids 35–154 when compared to human p21/Cip1, presented a very different picture (Fig. 2B). CMT12 cells contained p21/Cip1 cDNA sequences identical to that found in published human and chimpanzee p21/Cip1 sequences except for 1 silent third base difference (Fig. 2B, nt 9). When compared to the published canine p21/Cip1 sequence (Genbank No. AF076469, 26) CMT12-derived p21/Cip1 contained seven nucleotide changes that resulted in five amino acid differences four of which were conservative and one which was a non-conservative non-polar to polar amino acid change (Fig. 2B, nt 59, valine to glycine).

Amplicons encoding 295 bp of the p27/KIP1 transcript were the most conserved among dogs and humans with only two nucleotide differences between published canine (Genbank No. AY004255) and human (Genbank No. NM_004064) sequences resulting in a conservative glutamic acid to aspartic acid and non-conservative valine to glycine (non-polar to uncharged polar) amino acid changes (Fig. 2C, nt 30 and 291). The p27/KIP1 cDNA sequence isolated from CMT12 cells contained sequences identical to the human sequence at these positions.

Dendrograms comparing each of the three clusters of CKI sequences from dog with those from humans and other mammalian species clearly show clustering of canine and human/primate sequences for all three CKIs sequences (Fig. 3). Domestic chicken p27/KIP1 sequences were included for p27 alignments to root comparisons due to the high level of sequence conservation among mammals. Rodent and feline sequences were clustered separately suggesting a strong conservation of sequence between canine and human CKI coding sequences.

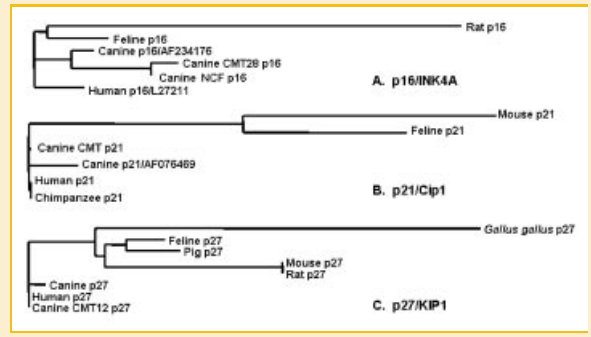


Fig. 3. Dendrograms of canine CKIs. A neighbor-joining algorithm (ClustalW—Vector NTI) was used to calculate a rooted relationship dendrogram for related sequences for each of p16/INK4A, p21/Cip1 and p27/KIP1 comparing published canine, human, rat or mouse, other mammals as noted (and including domestic chicken sequences for p27/KIP1) and all of the canine CMT cell line and NCF-derived CKI sequences. Genbank accession numbers are indicated (see legend to Fig. 2). A: p16/INK4A with calculations rooted on the rat p16/INK4A sequence. B: p21/Cip1 with calculations rooted on the mouse p16/INK4A sequence. C: p27/KIP1 with calculations rooted on the chicken p16/INK4A sequence (*Gallus gallus* AY094490).

## CHARACTERIZATION OF p16/INK4A EXPRESSION AND PHENOTYPE RESCUE IN TRANSFECTED CMT CELL CLONES

Phenotype-rescue of the transformed phenotype of CMT cells was evaluated following transfection of a eukaryotic expression construct containing a complete cDNA encoding human p16/INK4A and conferring resistance to Geneticin by a neomycin resistance cassette (Fig. 4A). Following transfection and selection in Geneticin, cells were subjected to single cell sorting into a 96-well plate and allowed to grow until colonies could be counted. Efficiency of cloning was used to estimate suppression of immortality within 6 weeks after cloning. Weekly counts of new colonies revealed comparable kinetics of growth for clones derived from all three CMT populations suggesting no immediate suppression of immortality (Fig. 4B). Cumulative efficiencies were also comparable. The cloning efficiency of single cell clones of CMT/p16 resulted in cloning efficiencies of 88–92% for all parental cell lines.

Expression of p16/INK4A was assessed by rt-PCR amplifications of single cell clones revealing abundant expression in most clones recovered from each CMT cell line (Fig. 4C). Analysis of the levels of p16/INK4A protein expression in these clones by Western blot (50 μg/lane of total proteins from each cell population) revealed trace to low levels of p16/INK4A in most clones comparable to the levels observed in NCF cells (Fig. 4D). Additionally, CMT12/p16 clones were re-evaluated (100 μg/lane of total protein from each cell population), with essentially identical results (data not shown). Occasionally, clones were recovered that were Geneticin resistant but that did not express p16/INK4A mRNAs or proteins (e.g., Fig. 4C, clone CMT12/p16B and Fig. 4D, CMT12/p16, lane B) which were used as p16/INK4A$^{-}$, neomycin-resistant (neo$^{r}$) control cultures in assays of proliferation kinetics performed in the presence of Geneticin (see below).

CMT cell lines developed changes in appearance and level of contact inhibition following transfection of the p16/INK4A *trans*-gene and subsequent selection (Fig. 5). CMT12/p16 clones exhibited the fewest changes with some cell enlargement evident (Fig. 5A). CMT27/p16 clones exhibited considerable cell enlargement without much change in shape from the stellate form of the parental line although exceptions were noted (Fig. 5B, compare clone G to other clones). As these cells reached confluence and became mature aged cultures (after approximately 1 week at confluence) super confluent layers of cells were evident in some cultures (Fig. 5B, clone A)

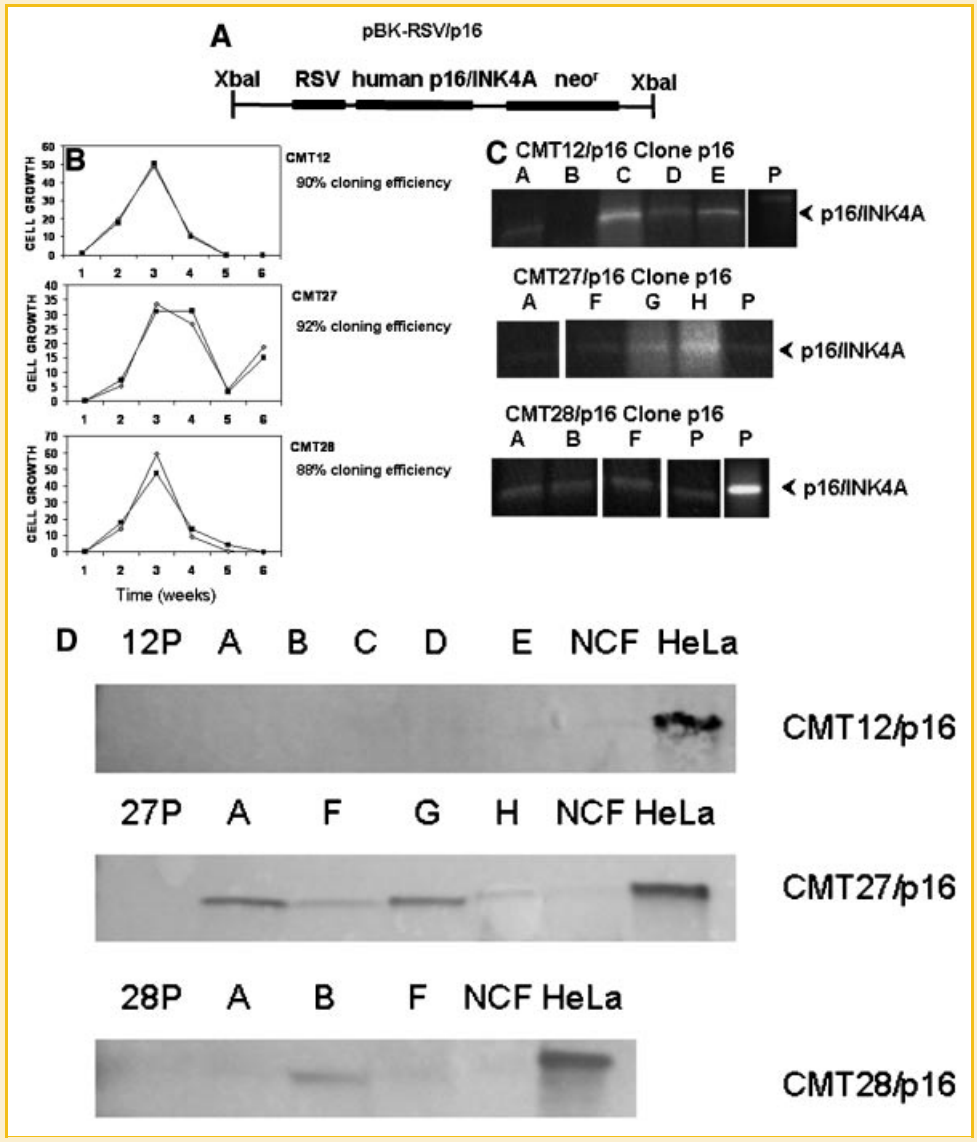


Fig. 4. Characterization of p16/INK4A expression following transfection of CMT cell clones. Phenotype-rescue of CMT cell clones was assessed after transfection of human p16/INK4A CKI expression constructs followed by selection and single cell cloning. A: Human expression clone of pBK-RSV/p16 encoding p16/INK4A [Koenig et al., 2002]. Linear plasmids, cut at the *Xba*I sites, were transfected into each parental CMT cell line. Cells were selected for neomycin resistance (neo$^r$) in the presence of G418. Human p16/INK4A expression was driven off of the RSV promoter. B: Cloning efficiency of p16/INK4A transfected CMT cell clones from three parental CMT cell lines. Transfected and G418 selected cells were single cell sorted into 96-well plates and scored for evidence of cell growth over 6 weeks. Plots were of cells on uncoated plastic (■) or gelatin-coated (◆) wells. Number of wells in which new evidence of cell proliferation (presence of multiple cells) was observed was recorded at each time point. In all cases cumulative cloning efficiency was estimated at 88–92% for all parental cell lines which eventually produced recognizable colonies. C: Semi-quantitative rt-PCR of p16/INK4A transcripts in CMT cells and transfected clones. Several clonal colonies derived from each CMT parent cell line (P) were randomly selected and were designated with upper case capital letters (A, B, C, etc.) and then a selection of these lines were used for further characterization. For rt-PCR analysis, 1 μg of total RNA was amplified and 15 μl/lane loaded for each cell clone analyzed by agarose gel electrophoresis. The position of the p16/INK4A amplicon is noted (arrows). Clonal cell lines that failed to express p16/INK4A transgene were also selected as G418-resistant negative controls (CMT12/p16B). D: Western blots of p16/INK4A in CMT parental cells (P) and transfected clones (A, B, C, etc.) as well as selected human tumor cells (HeLa) and NCF cells.

while others appeared to have re-acquired some contact inhibition (Fig. 5B, clone H). A few clones failed to proliferate beyond approximately 10 passages following recovery from cloning plates at 6 weeks suggesting a reacquisition of limited life-span and senescent cell death (Fig. 5B, clone F). CMT28/p16 clones also exhibited these changes including a pronounced improvement in contact inhibition as well as altered confluent associations of more parallel and aligned cultures once confluence was achieved (Fig. 5C, clones B and F). CMT28/p16 clones exhibited altered cell morphologies in that clone A was more spindle shaped while clone B and clone F were more stellate with the latter exhibiting a smaller cell size than the parental cells.

Direct comparison of the effects of exogenous p16/INK4A expression on proliferative velocity was achieved by direct comparison of transfected clones to each other and parental strains in cell growth kinetic analyses (Fig. 6). In CMT12 and CMT27 cells, defective in p16/INK4A expression, p16/INK4A transgene expression clearly decreased the rate of proliferation in most transfected clonal cell lines. Exceptions that proliferated at rates comparable to parental lines were noted such as CMT12/p16B in which no p16/INK4A expression was evident despite clear neomycin resistance. Other clones also proliferated at rates comparable to parental lines although most clones analyzed proliferated at rates slower than parental lines assessed as the slope of the line in cell proliferation plots. For all three CMT lines parental CMT cells were always among the more rapidly proliferating populations. Two different control populations were employed in this analysis. Parental CMT12 cells were included as controls but because they are not resistant to Geneticin and were cultured in antibiotic-free medium, an additional control was included. CMT12/p16B cells were Geneticin resistant but did not express the transgene and were also employed as p16/INK4A-untransfected neomycin-resistant controls to compare the effects of the transgene in other clones in the presence of Geneticin. No differences in the proliferation rates of these two different control populations were noted. In contrast to those parental CMT lines defective in p16/INK4A expression, CMT28 cells

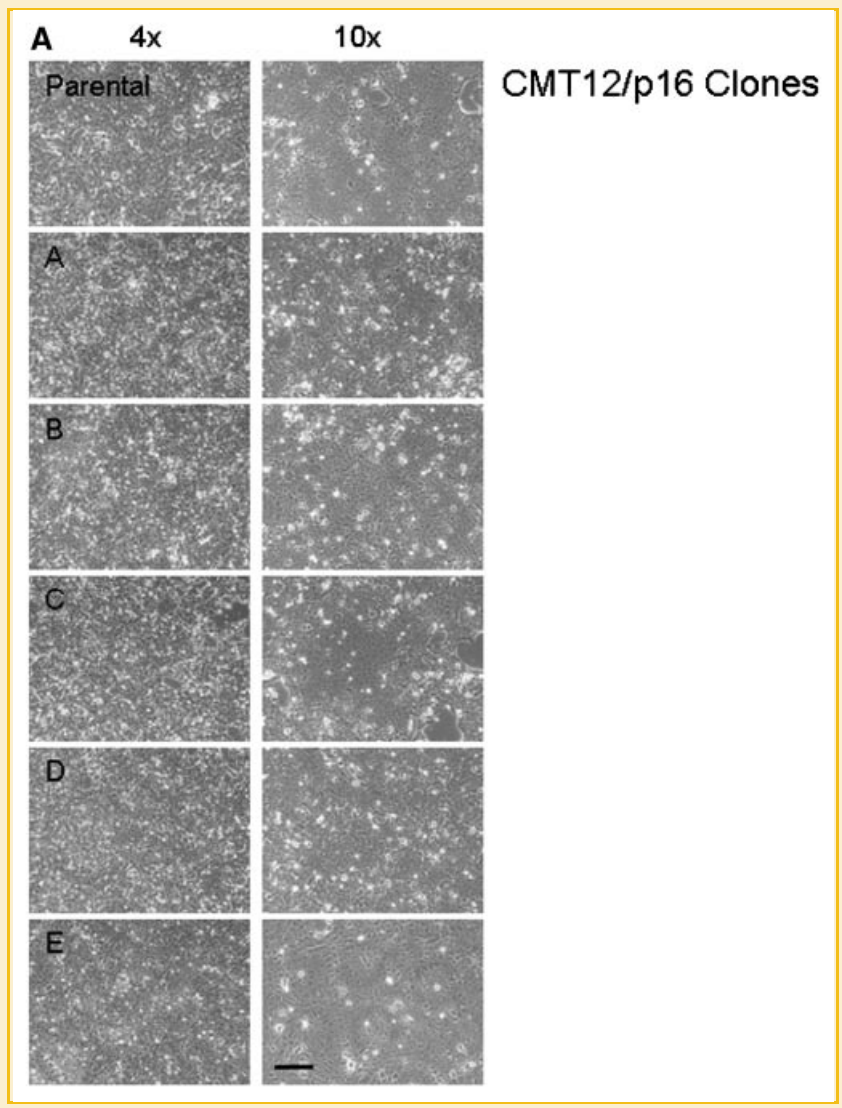


Fig. 5. Phenotype of p16/INK4A transfected CMT cell clones and parental cell lines. A: Live cultures of selected CMT12/p16 clones (A–E) and parental cells were examined under phase-contrast optics using a 4× and 10× objective (magnification bar indicates scale for each) and photographed. Representative fields of cells are shown for each culture. B: Live cultures of selected CMT27/p16 clones (A, F–H) and parental cells. Cultures are shown when cell growth had achieved low confluence, higher confluence and older confluence (see Materials and Methods for details) at magnifications noted. C: Live cultures of selected CMT28/p16 clones (A, B, F) and parental cells. Cultures are shown when cell growth had achieved relative confluence as described above. Normal canine fibroblasts (NCFs) at subconfluent cell density are shown for comparison. Scale bars equal 200 μm.

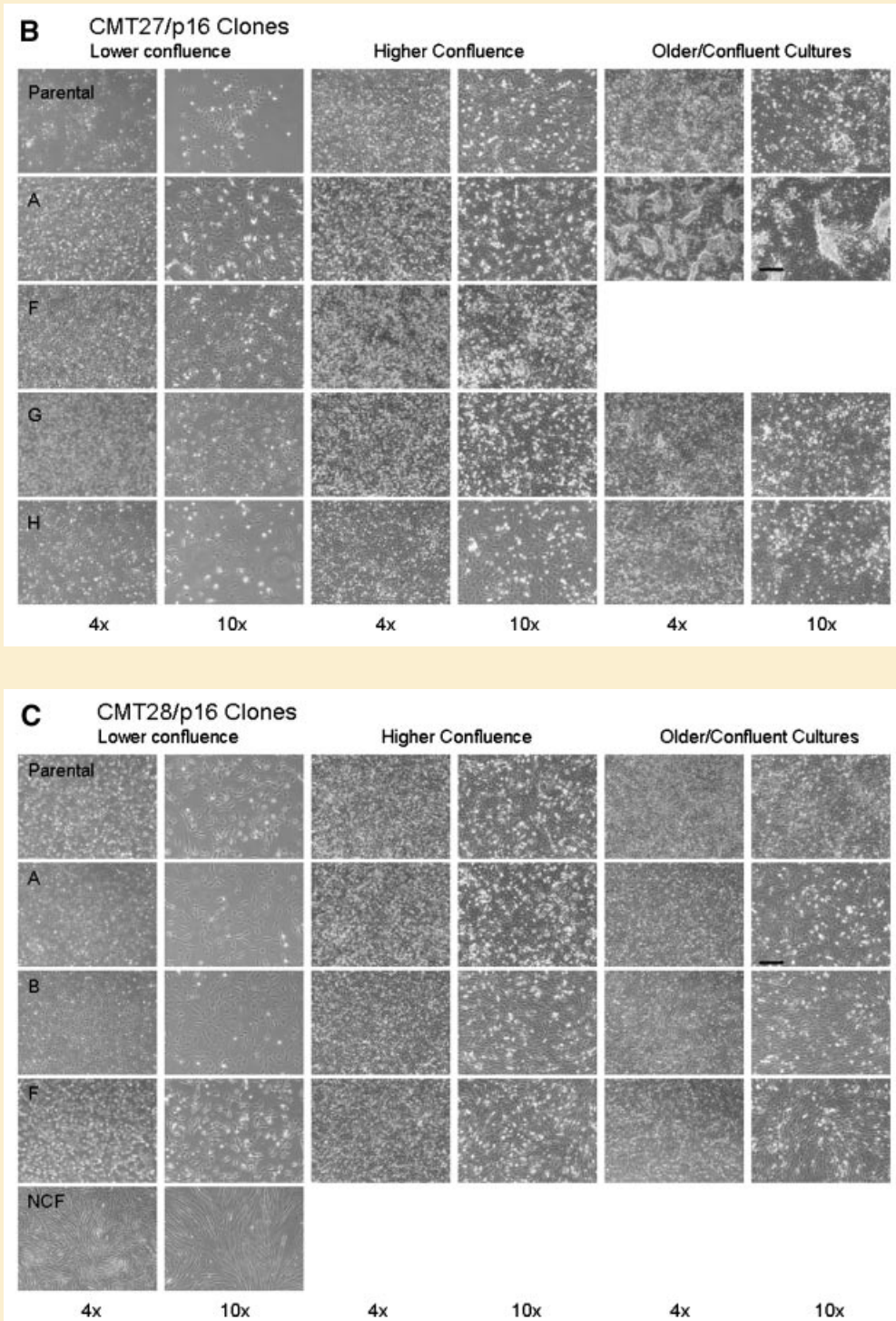


Fig. 5. (*Continued*)

which expressed p16/INK4A, were essentially unchanged in proliferation rate by p16/INK4A transfection.

To determine if changes in proliferative velocity were caused by alterations in cell cycle phase distribution, flow cytometry was used to analyze propidium iodide stained DNA content in parental CMT cell lines and p16/INK4A transfected clones derived from them (Fig. 7). Some lengthening of G2/M phase was noted in transfected clones of CMT12 compared to parental cells. Small apparent lengthening of G1 phase was noted in the cell cycle phase distributions of CMT27 and CMT28 parental cells and transfected

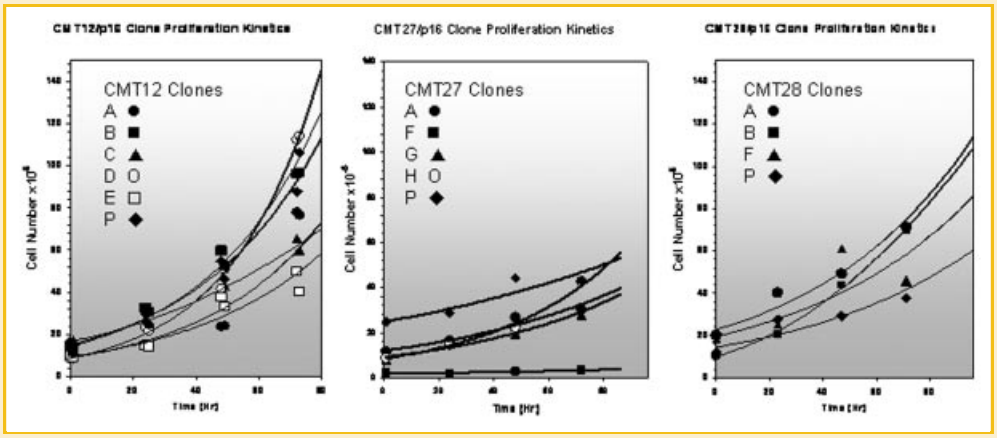


Fig. 6. Cell proliferation kinetics of p16/INK4A transfected CMT cell clones and parental cell lines. Cultures of each parental and each clonal cell line were grown for 70 h or more and cell counts determined in replicate wells. Cell counts were plotted against time (h) in complete growth medium containing G418 except for parental lines in which G418 was omitted. A: CMT12/p16 clones (clone A ●, B ■, C ▲, D ○, E □) and parental CMT12 (◆) cells. B: CMT27/p16 clones (clone A ●, F ■, G ▲, H ○) and parental CMT27 (◆) cells. By approximately 25 passages clone CMT27/p16F appeared to become senescent because it failed to proliferate. C: CMT28/p16 clones (clone A ●, B ■, F ▲) and parental CMT12 (◆) cells.

clones derived from them suggesting cell cycle was only modestly perturbed by the transgene. Proliferative velocity was thus most likely a result of increased speed of cell cycle transit generally and not due to changes in duration of an individual cell cycle phase. Importantly, by approximately 25 passages in some transfected clones such as CMT27/p16F, these clones appeared to become senescent because they failed to proliferate.

To evaluate the ability of CMT cell lines and transfected cell clones derived from them to grow in the absence of substrate, a clonogenic assay was used to evaluation substrate-dependence of cell growth in soft agar (Fig. 8). Transfection of p16/INK4A expression constructs into CMT cells resulted in very different alterations to substrate dependence of growth apparently due to differences in the original parental cell lines. Despite being

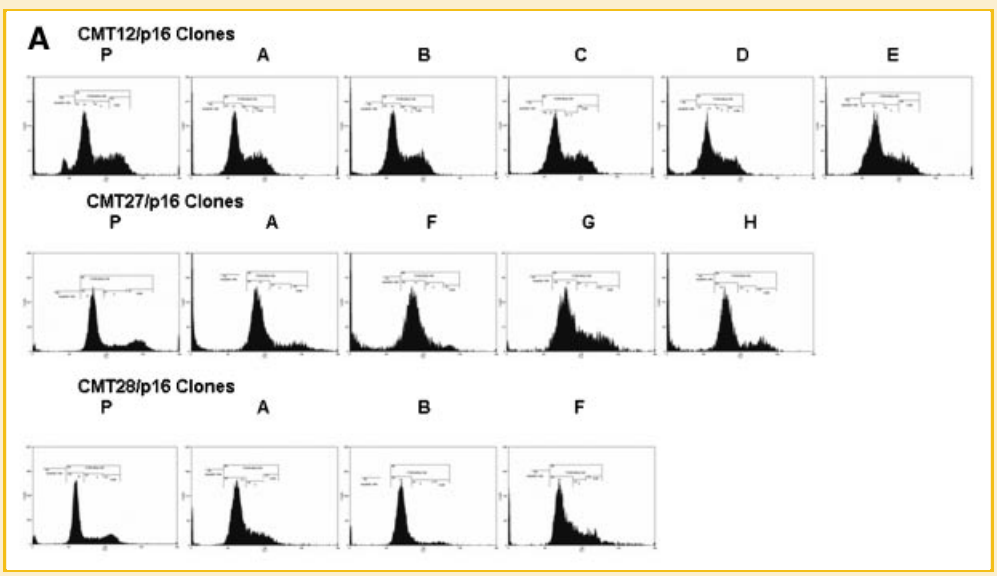


Fig. 7. Cell cycle profile analysis of p16/INK4A transfected CMT cell clones and parental cell lines. Subconfluent (<70%) parental CMT and p16/INK4A-transfected cell clones were fixed and stained with propidium iodide and analyzed for cell cycle phase by flow cytometry on a linear fluorescent scale. The percentage of each population in each phase of the cell cycle (G1, S and G2/M phases) were determined as were the proportion of apoptotic cells in each proliferating culture. A: CMT12/p16, CMT27/p16, and CMT28/p16 clones (as noted for each cell line in Fig. 6) and parental cell lines (P). B: Relative cell cycle phase distribution of p16/INK4A transfected CMT cell clones and parental cell lines were determined (percentage of the non-apoptotic cell population) for CMT12/p16, CMT27/p16, and CMT28/p16 cell clones and parental cell lines. For each parental and clonal cell line relative percentage of each population in G1 (black bars), S (gray bars) and G2/M (white bars) phases are shown.

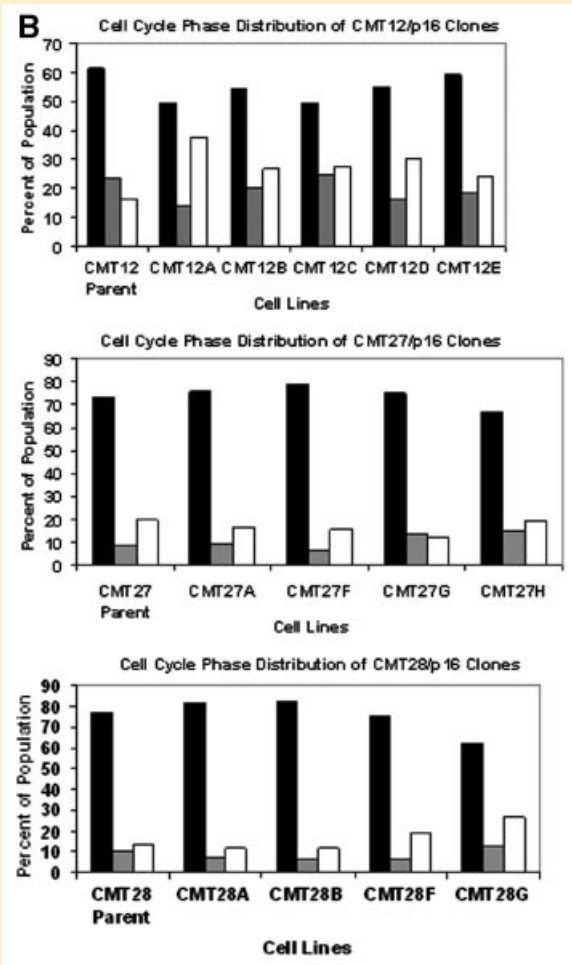


Fig. 7. (*Continued*)

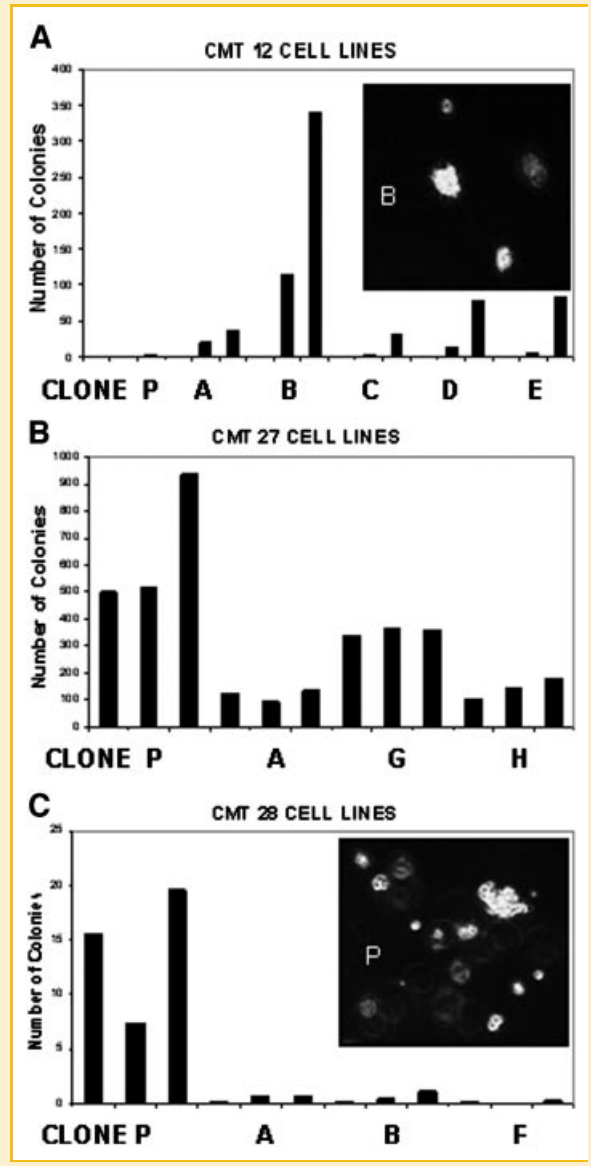


Fig. 8. Substrate-dependent growth analysis by soft-agar assay of p16/INK4A transfected CMT cell clones and parental cell lines. CMT parental cell (P) and p16-transfected cell clones (as noted in Fig. 6) were grown in soft agar in complete growth medium and allowed to proliferate to form colonies. Each assay was performed in triplicate (three bars per line as shown) and the experiment was performed at least three times. Number of colonies evident per well were determined. A: CMT12/p16 transfected cell clones and parental cell lines. B: CMT27/p16 clones transfected cell clones and parental cell lines. C: CMT28/p16 clones transfected cell clones and parental cell lines. Inset, representative phase contrast images of multi-cell colonies growing in soft agar medium.

transformed, CMT12 cells still retained measurable substrate dependence for growth and subsequent transfection and selection actually resulted in recovery of clones that reflected low levels of enhancement of clonogenic activity in soft agar in some clones compared to parental cells. These results were contrary to predicted outcomes and likely reflect inherent heterogeneity in the parental cell line. In contrast, parental CMT27 and CMT28 cells were both highly clonogenic in soft agar and this phenotype was reversed/suppressed by transfection of the p16/INK4A transgene. This was true in CMT28 even though CMT28 cells expressed abundant endogenous p16 protein and did not experience an apparent rescue of transformation when other characteristics were assayed.

## DISCUSSION

### CANCER AND THE DEREGULATION OF CKIs

The CKI p16/INK4A has been classified as a tumor suppressor gene as it is inactivated in up to 85% of tumor-derived cell lines and is the second most common mutation found in cancer [Pavletich, 1999; Tyner and Gartel, 2003]. Because a majority of tumors are carcinomas that require re-entry into the cell cycle for tissue replacement, there is a rationale for linking cancer development with interference in CKI function [Tyner and Gartel, 2003]. Similarly, other pathways involving CKI function are frequently altered or deregulated in spontaneous cancers [Pavletich, 1999]. DNA repair-mediated arrest of cell cycle in G1 phase appears to be

the result of p53 activation and p53 mutations are the most commonly observed defects in human cancers. Additionally, p53 loss results in the inability to arrest cell cycle because the CKI p21/Cip1 is a target for transcription activation by p53 [Levine, 1997; Schafer, 1998]. Similarly, DNA damaging radiation fails to arrest cell cycle progression in p53 knockout mice because activation of p21/CIP transcription fails [Little et al., 1995; Waldman et al., 1995; Yao et al., 1996]. p27 expression can also be abnormally regulated in cancer and CDK4 mutations that fail to respond to p16/INK4A suppression have been observed [Wolfel et al., 1995; Sandhu and Slingerland, 2000]. Thus, significant variation exists in the types of CKI-pathway defects observed in cancer perhaps due to the redundancies built into this system. Understanding how CKI tumor suppressor gene defects promote cancer phenotypes represents a key and common requirement for understanding neoplastic mechanisms.

Our initial analysis of CKI expression in CMT cells suggested such heterogeneity was also evident in these lines derived from spontaneous CMTs. This is consistent with our previous detection of SIRT2 expression in each of these cell lines and defects in p53 and p21/Cip1 expression in CMT28 cells [DeInnocentes et al., 2006]. Although for CMT28 cells, p16/INK4A expression was at normal to somewhat induced levels, both CMT12 and CMT27 cells were found to be defective in p16/INK4A expression. Thus, each of the CMT cell lines investigated were defective in one or more CKI tumor suppressor genes. Either the p16/INK4A and Rb pathway was defective suggesting a defect in cell cycle exit or the p53/p21 pathway was defective suggesting apoptotic signaling may be inoperative [Medema et al., 1995; Bird, 2008]. Coupled to previously identified c-*erb*B-2 overexpression in all three cell lines and the potential for c-*yes* oncogene activation in such canine tumors, we have proposed a putative model for spontaneous canine mammary cancer [Miyoshi et al., 1991; Ahern et al., 1996; Rungsipipat et al., 1999; Bird, 2008]. Such detailed genetic characterization places these cell lines and canine mammary cancers as highly comparable intermediate models between murine and human breast cancers (Table I).

## NEOPLASTIC PHENOTYPE RESCUE/REVERSAL BY p16/INK4A TRANSFECTION

Based on this data, we investigated the potential of gene replacement of p16/INK4A in phenotype rescue/reversal of transformation in CMT cells that do not express this important tumor suppressor gene. High levels of homology between human and canine sequences strongly suggested that function would be conserved and that human p16/INK4A could be reasonably expected to compliment defects in canine expression [Koenig et al., 2002]. These experiments were designed to correct defects in the CMT cell line by complementation in *trans*. All 3 CMT cell lines, including the two that appear defective in p16/INK4A expression and one that appeared normal or slightly induced, were permanently transfected with human p16/INK4A cDNA cloned into an expression vector that also expressed neomycin resistance [Koenig et al., 2002]. This construction allowed selection of transfectants followed by single cell cloning to allow comparison of individual gene insertion events. Not all clones were capable of proliferation, however, most (88–92%) grew and were eventually expanded into sufficient numbers in which p16/INK4A expression could be assayed and proliferation kinetics assessed against parental controls and a clone that expressed neomycin resistance but not p16/INK4A. Assays of neoplastic character were then assessed to determine if transfection of p16/INK4A activity affected the phenotype of CMT cells defective in p16/INK4A expression. Assays for neoplastic phenotype included visual assessment of changes in cell phenotypes, assessment of loss of contact inhibition in confluent cultures, altered/lower rates of cell proliferation compared to parental cell lines and neomycin resistant/p16/INK4A defective cell lines, induction of senescence reversing immortal or extended lifespan, and assessment of substrate dependence of cell growth. Assessment of tumorigenicity in immune-compromised mice was not assessed as these cell lines were not tumorigenic under such circumstances [Sartin et al., 1993; Ahern et al., 1996].

A series of clonal cell lines expressing p16/INK4A, derived from each parental cell line, demonstrated a clear association in most cases between p16/INK4A expression and reduced proliferation rates. Additionally, cell phenotype including contact inhibition which was enhanced as was induction of a more normal/non-neoplastic phenotype, was also associated with enhanced p16/INK4A expression. This was particularly clear for at least one cell line, CMT27F/p16, in which the apparent induction of a senescent phenotype was achieved. Thus, transfection of p16/INK4A was able, in this clone, to rescue the transformed phenotype allowing these cells to become senescent with the loss of proliferative potential followed by arrest. This demonstrated that p16/INK4A transfection has the capacity to at least partially repair/rescue the transformed phenotype in CMT cells.

We also assessed the dependence of each parental and clonal cell line to proliferate in the absence of a solid substratum. Normal cells grow and make colonies poorly in the absence of contact with a solid surface while neoplastic cells frequently have lost their dependence on a solid substratum for proliferation. Colony forming rates in the absence of substrate determined for parental cell lines were predicted to be suppressed in clonal cell lines transfected with p16/INK4A. Both CMT27 and CMT28 parental cells were highly clonogenic and proliferative in soft-agar assays demonstrating substrate independent proliferation. In contrast and as predicted, p16/INK4A-transfected clones demonstrated a uniform suppression of this characteristic as p16/INK4A transfection rescued substrate-dependent growth in both cell lines. Transfected CMT12 clones were, in contrast, more substrate-independent and more variable than the parental line which had retained measurable substrate-dependence for growth. This may have been the result of unveiling underlying heterogeneity in the parental population through single cell cloning or could reflect a real enhancement of substrate independence of growth. Although the results in CMT12 derived cells were less easily explained, the similarity of the effects of phenotype rescue in CMT27 and CMT28 cells suggested that, despite detection of ample p16/INK4A expression in parental CMT28 cells, this pathway was likely defective in CMT28 cells as well as they appeared rescued by transfection of p16/INK4A. For all characteristics of transformation evaluated, the transformed phenotype was reversed or substantially rescued at least in some clones derived from all three cell lines.

### AN INTERMEDIATE CANINE BREAST CANCER MODEL

Canine cancers are proving to be valuable intermediate models of human cancers bridging the biological distance between induced mouse models and spontaneous human malignancies [Khanna et al., 2006; Bird et al., 2008]. CMT cells provide an excellent cellular model of human breast cancers derived from spontaneous canine mammary cancers with measurable changes/defects in expression of genes known to regulate cell proliferation and cell cycle entry/exit. These include defects in tumor suppressor genes encoding CKIs p16/INK4A and p21/Cip1 as well as p53 expression defects and oncogene activations including *erb*B-2 and c-*yes* [Miyoshi et al., 1991; Medema et al., 1995; DeInnocentes et al., 2006; Bird et al., 2008]. These analyses, while not exhaustive, have provided a much more comprehensive picture of the gene expression profile of each of these unique cell lines. The gene defects that have been described demonstrate a high level of similarity to defects found in human breast cancer and further support development of this unique intermediate animal model for development of new therapeutic strategies [Bird et al., 2008].

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## REFERENCES

Ahern TE, Bird RC, Church Bird AE, Wolfe LG. 1996. Overexpression of the oncogene c-erbB-2 in canine mammary carcinomas and tumor-derived cell lines. Am J Vet Res 57:693–696.

Arellano M, Moreno S. 1997. Regulation of CDK/cyclin complexes during the cell cycle. Int J Biochem Cell Biol 29:559–573.

Bird RC. 1997. The cyclin/cyclin-dependent kinase (cdk) complex: Regulation of cell cycle progression and nuclear disassembly. In: Bird RC, Stein GS, Lian JB, Stein JL, editors. Nuclear structure and gene expression. San Diego: Academic Press. pp 145–174.

Bird RC. 2003. Regulation of cyclin-dependent kinase 1 (cdc2) expression during the G0/G1 phase transition and the consequences for regulation of cell cycle progression. In: Boonstra J, editor. G1 phase progression. Georgetown: Landes Biosciences. pp 1–18.

Bird RC, DeInnocentes P. 2004. Characterization of the CDP-Like/CTAS-1 Binding Site in the Okadaic Acid Response Element (OARE) of the Human CDK1($p34^{cdc2}$) Promoter. Anticancer Res 24:1469–1480.

Bird RC, DeInnocentes P, Lenz S, Thacker EE, Curiel DT, Smith BF. 2008. Cross-presentation of a hybrid-cell fusion vaccine against canine mammary cancer. Vet Immunol Immunopathol 123:289–304.

Bird RC. 2008. Defects in genes regulating the cell cycle in spontaneous canine models of cancer. In: Yoshida K, editor. Trends in cell cycle research. Kerala: Research Sign Post (in press).

Bostrom J, Meyer-Puttlitz B, Wolter M, Blaschke B, Weber RG, Lichter P, Ichimura K, Collins VP, Reifenberger G. 2001. Alterations of the tumor suppressor genes CDKN2A (p16(INK4a)), p14(ARF), CDKN2B (p15(INK4b)), and CDKN2C (p18(INK4c)) in atypical and anaplastic meningiomas. Am J Pathol 159:661–669.

Carnero A, Hudson JD, Price CM, Beach DH. 2000. p16INK4A and p19ARF act in overlapping pathways in cellular immortalization. Nat Cell Biol 2:148–155.

Cheng T, Scadden DT. 2002. Cell cycle entry of hematopoietic stem and progenitor cells controlled by distinct cyclin-dependent kinase inhibitors. Int J Hematol 75:460–465.

Christopher SA, Diegelman P, Porter CW, Kruger WD. 2002. Methylthioadenosine phosphorylase, a gene frequently codeleted with p16(cdkN2a/ARF), acts as a tumor suppressor in a breast cancer cell line. Cancer Res 62:6639–6644.

DeInnocentes P, Li LX, Sanchez RL, Bird RC. 2006. Expression and sequence of canine SIRT2 and p53 alleles in canine mammary tumor cells—Effects on down stream targets Wip1 and p21/Cip1. Vet Comp Oncol 4:161–177.

Ekholm SV, Reed SI. 2000. Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. Curr Opin Cell Biol 12:676–684.

Forsburg SL, Nurse P. 1991. Cell cycle regulation in the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe. Annu Rev Cell Biol 7:227–256.

Frey RS, Li J, Singletary KW. 2001. Effects of genistein on cell proliferation and cell cycle arrest in nonneoplastic human mammary epithelial cells: Involvement of Cdc2, p21waf/cip1, p27kip1, and Cdc25C expression. Biochem Pharmacol 61:979–989.

Hamburger AW, Salmon SE. 1977. Primary bioassay of human tumor stem cells. Science 197:461–463.

Jeffrey PD, Tong L, Pavletich NP. 2000. Structural basis of inhibition of CDK-cyclin complexes by INK4 inhibitors. Genes Dev 14:3115–3125.

Khanna C, Lindblad-Toh K, Vail D, London C, Bergman P, Barber L, Breen M, Kitchell B, McNeil E, Modiano JF, Niemi S, Comstock KE, Ostrander E, Westmoreland S, Withrow S. 2006. The dog as a cancer model. Nat Biotechnol 24:1065–1066.

Koenig A, Bianco SR, Fosmire S, Wojcieszyn J, Modiano JF. 2002. Expression and Significance of p53, Rb, p21/waf-1, p16/ink-4a, and PTEN Tumor Suppressors in Canine Melanoma. Vet Pathol 39:458–472.

LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E. 1997. New functional activities for the p21 family of CDK inhibitors. Genes Dev 11:847–862.

Levine AJ. 1997. p53 the cellular gatekeeper for growth and division. Cell 88:323–331.

Little JB, Nagasawa H, Keng PC, Yu Y, Li CY. 1995. Absence of radiation-induced G1 arrest in two closely related human lymphoblast cell lines that differ in p53 status. J Biol Chem 270:11033–11036.

Liu H, Bird RC. 1998. Characterization of the enhancer-like okadaic acid response element region of the cyclin-dependent kinase 1 (p34cdc2) promoter. Biochem Biophys Res Commun 246:696–702.

MacEwen EG, Withrow SJ. 1996. Tumors of the mammary gland. In: Withrow SJ, MacEwen EG, editors. Small animal oncology. Philadelphia: Saunder Co. pp 356–372.

Malek NP, Sundberg H, McGrew S, Nakayama K, Kyriakides TR, Roberts JM. 2001. A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. Nature 413:323–327.

Malumbres M, Perez De Castro I, Hernandez MI, Jimenez M, Corral T, Pellicer A. 2000. Cellular response to oncogenic ras involves induction of the Cdk4 and Cdk6 inhibitor p15(INK4b). Mol Cell Biol 20:2915–2925.

Medema RH, Herrera RE, Lam F, Weinberg RA. 1995. Growth suppression by p16INK4A requires functional retinoblastoma protein. Proc Natl Acad Sci USA 92:6289–6293.

Migone F, Bird RC, DeInnocentes P, Lenz S, Smith B. 2006. Alterations in CDK1 expression and localization following induction in a spontaneous canine mammary cancer model. J Cell Biochem 98:504–518.

Miyoshi N, Tateyama S, Ogawa K, Yamaguchi R, Kuroda H, Yasuda N, Shimizu T. 1991. Abnormal structure of the canine oncogene, related to

the human c-yes-1 oncogene, in canine mammary tumor tissue. Am J Vet Res 52:2046–2049.

Nakayama K, Nakayama K. 1998. Cip/Kip cyclin-dependent kinase inhibitors: Brakes of the cell cycle engine during development. Bioessays 20:1020–1029.

Ritt MG, Mayor J, Wojcieszyn J, Smith R, Barton CL, Modiano JF. 2000. Sustained nuclear localization of p21/WAF-1 upon growth arrest induced by contact inhibition. Cancer Lett 158:73–84.

Sandhu C, Peehl DM, Slingerland J. 2000. p16INK4A mediates cyclin dependent kinase 4 and 6 inhibition in senescent prostatic epithelial cells. Cancer Res 60:2616–2622.

Sartin EA, Barnes S, Toivio-Kinnucan M, Wright JC, Wolfe LG. 1993. Heterogenic properties of clonal cell lines derived from canine mammary carcinomas and sensitivity to tamoxifen and doxorubicin. Anticancer Res 13:229–236.

Sherr CJ, Roberts JM. 1999. CDK inhibitors: Positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512.

Pavletich NP. 1999. Mechanisms of cyclin-dependent kinase regulation: Structures of Cdks, their cyclin activators, and Cip and INK4 inhibitors. J Mol Biol 287:821–828.

Ruas M, Peters G. 1998. The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1378:F115–F177.

Rungsipipat A, Tateyama S, Yamaguchi R, Uchida K, Murakami Y, Miyoshi N, Hayashi T. 1999. Amplification of the c-yes oncogene in canine mammary tumors. J Vet Med Sci 61:185–189.

Russo AA, Tong L, Lee J-O, Jeffrey PD, Pavletich NP. 1998. Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumor suppressor p16INK4a. Nature 395:237–243.

Sandhu C, Slingerland J. 2000. Deregulation of the cell cycle in cancer. Cancer Detect Prev 24:107–118.

Schafer KA. 1998. The cell cycle: A review. Vet Pathol 35:461–478.

Sharpless NE, DePinho RA. 1999. The INK4A/ARF locus and its two gene products. Curr Opin Genet Dev 9:22–30.

Tyner AL, Gartel AL. 2003. Roles of cyclin kinase inhibitors in G1 phase progression. In: Boonstra J, editor. G1 phase progression. Georgetown: Landes Biosciences. pp 40–57.

Smits VA, Klompmaker R, Vallenius T, Rijksen G, Mäkela TP, Medema RH. 2000. p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint. J Biol Chem 275:30638–30643.

Waldman T, Kinzler KW, Vogelstein B. 1995. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res 55:5187–5190.

Wei W, Hemmer RM, Sedivy JM. 2001. Role of p14(ARF) in replicative and induced senescence of human fibroblasts. Mol Cell Biol 21:6748–6757.

Wolfe LG, Buxton-Smith B, Toivio-Kinnucan MA, Sartin EA, Kwapien RP, Henderson RA, Barnes S. 1986. Biologic properties of cell lines derived from canine mammary carcinomas. J Natl Cancer Inst 77:783–792.

Wolfel T, Hauer M, Schneider J, Schneider J, Serrano M, Wölfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Büschenfelde KH, Beach D. 1995. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 269:1281–1284.

Yao SL, Akhtar AJ, McKenna KA, Bedi GC, Sidransky D, Mabry M, Ravi R, Collector MI, Jones RJ, Sharkis SJ, Fuchs EJ, Bedi A. 1996. Selective radiosensitization of p53-deficient cells by caffeine-mediated activation of p34cdc2 kinase. Nat Med 2:1140–1143.

You J, Bird RC. 1995. Selective induction of cell cycle regulatory genes cdk1 (p34cdc2), cyclins A/B, and the tumor suppressor gene Rb in transformed cells by okadaic acid. J Cell Physiol 164:424–433.

Zhang P, Wong C, Liu D, Finegold M, Harper JW, Elledge SJ. 1999. p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. Genes Dev 13:213–224.