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Tumor Suppressor Gene p16/INK4A/CDKN2A-Dependent Regulation Into and Out of the Cell Cycle in a Spontaneous Canine Model of Breast Cancer

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ABSTRACT

p16/INK4A/CDKN2A is an important tumor suppressor gene that arrests cell cycle in G1 phase inhibiting binding of CDK4/6 with cyclin D1, leaving the Rb tumor suppressor protein unphosphorylated and E2F bound and inactive. We hypothesized that p16 has a role in exit from cell cycle that becomes defective in cancer cells. Well characterized p16-defective canine mammary cancer cell lines (CMT28, CMT27, and CMT12), derived stably p16-transfected CMT cell clones (CMT27A, CMT27H, CMT28A, and CMT28F), and normal canine fibroblasts (NCF), were used to investigate expression of p16 after serum starvation into quiescence followed by re-feeding to induce cell cycle re-entry. The parental CMT cell lines used lack p16 expression either at the mRNA or protein expression levels, while p27 and other p16-associated proteins, including CDK4, CDK6, cyclin D1, and Rb, were expressed. We have successfully demonstrated cell cycle arrest and relatively synchronous cell cycle re-entry in parental CMT12, CMT28 and NCF cells as well as p16 transfected CMT27A, CMT27H, CMT28A, and CMT28F cells and confirmed this by ^{3}H-thymidine incorporation and flow cytometric analysis of cell cycle phase distribution. p16-transfected CMT27A and CMT27H cells exited cell cycle post-serum-starvation in contrast to parental CMT27 cells. NCF, CMT27A, and CMT28F cells expressed upregulated levels of p27 and p16 mRNA, post-serum starvation, as cells exited cell cycle and entered quiescence. Because quiescence and differentiation are associated with increased levels of p27, our data demonstrating that p16 was upregulated along with p27 during quiescence, suggests a potential role for p16 in maintaining these non-proliferative states. J. Cell. Biochem. 114: 1355–1363, 2013.

KEY WORDS: p16; CANINE MAMMARY CANCER; BREAST CANCER; CELL QUIESCENCE; CELL CYCLE

Cell cycle progression is controlled principally by cyclins and cyclin-dependent kinases (CDKs) and their cofactors activating them to promote cell cycle progression [Pines and Hunter, 1991]. Along with cyclins and CDKs, other associated proteins, such as the tumor suppressors Rb and p53 and transcription factors such as the E2F proteins, also play important roles in regulating cell cycle progression. Cyclin/CDK regulation is controlled by the activities of two important classes of such genes, which play critical roles in regulating transitions into and out of the cell cycle and may function as a gateway to terminal differentiation. At cell cycle checkpoints CDKs can be inhibited by cyclin-dependent kinase inhibitors (CKIs), inhibiting cell cycle progression [Kamb et al., 1994]. There are two important CKI families; the INK4 and KIP/CIP families [Swellam et al., 2004]. The INK4 family inhibitors inhibit CDK4 and CDK6 in association with cyclin D, while KIPs inhibit CDK2 and CDK4 in association with cyclin D and cyclin E. The INK4 family consists of p16/INK4A, p15/INK4B, p18/INK4C, and p19/INK4D, while the KIP family consists of p21/CIP1, p27/KIP1, and p57/KIP2 [Canepa et al., 2007]. All the CKIs are proven tumor suppressor genes or suspected of having this potential. Additionally, the INK4 family members are structurally similar, equally potent, and may have cell lineage-specific or tissue-specific functions [Canepa et al., 2007]. Expression

Conflicts of interest: Nothing to declare.

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of some members of the INK4 gene family, such as p18 and p19, appear to predominate during early-mid gestation in mouse development while expression of p15 has been found in later stages of gestation [Herman et al., 1997]. Thus, different INK4 proteins may not be redundant as they appear to be differentially expressed during development.

p16/INK4A/CDKN2A regulates cell cycle in early G1 phase stopping further transition from G1 to S phase as a component of a multi-protein regulatory complex. During G1 phase, CDK4 and CDK6 form complexes with cyclin D1 which in turn phosphorylate Rb resulting in additional Rb phosporylation by cyclin E/CDK complexes until there is release of the E2F family transcription factors from Rb/E2F complexes. Rb otherwise inhibits E2F suppressing further cell cycle progression as E2F initiates transcription of genes required for S phase [Bean et al., 2007; Sun et al., 2007]. The action of p16 inhibits binding of CDK4/6 to cyclin D1, which leaves Rb unphosphorylated and E2F bound and inactive [Vidal and Koff, 2000]. p16/INK4A protein causes inhibitory structural changes to CDK4 and CDK6 targets and can actually compete with cyclin D1 for CDK binding by blocking activating structural changes to bound CDKs. Binding of p16 results in changes in conformation of CDK proteins so that they can not bind cyclin D1 [Sharma et al., 2007]. p16 may also bind to pre-assembled CDK4/6-cyclin D1 complexes blocking their function.

p16 expression changes are highly correlated with changes affecting cell proliferation. In a coordinated way, maintenance of p16 and p27 CKI levels contribute to low levels of proliferation in normal blood vessels [Weinberg, 1995] and p16 mRNA and proteins accumulate in human fibroblasts as they become senescent [Lukas et al., 1996]. Deletion of the human 9p21 chromosome region, encoding p16, is also associated with tumor formation in a wide range of tissues [Kamb et al., 1994]. Loss of heterozygosity [Swellam et al., 2004], loss of homozygosity [Vidal and Koff, 2000; Izzard et al., 2002], and hypermethylation of the promoter [Tripathy and Benz, 1992; Hara et al., 1996] in the 9p21 region are all important mechanisms which have been shown to result in loss of p16 expression. Evaluation of p16 expression and function have been proposed as prognostic indicators in predicting breast cancer recurrence as p16 defects are second in frequency only to those in p53 for human malignancies [Baylin et al., 1998]. p16 gene deletions are significantly associated with late-stage, high-grade cancers [Morgan, 1997].

Cells are directed toward four possible fates following cell cycle exit: senescence, quiescence, differentiation, or death. Quiescence is the temporary non-proliferating stage characterized by an absence of DNA synthesis, lower metabolism, and smaller cell size [Yusuf and Fruman, 2003]. Differentiation is the developmental process through which cells gradually restrict their fate to a single terminal post-mitotic cell type. Senescence is the permanent exit of cells from cell cycle. Senescent cells cannot revert back to normal cell division under any physiological conditions. Unlike quiescent cells, senescent cells also exhibit a senescent phenotype including a large flattened cell shape with higher levels of metabolism and characteristic expression of β-galactosidase [Cristofalo et al., 2004]. p16 may play a key regulatory role in cell quiescence, differentiation and senescence through management of cell cycle exit. p16 induces and maintains the senescent phenotype [Stein et al., 1999] and has been reported capable of promoting premature cell senescence [Zindy et al., 1997]. In contrast, little is known about the role of p16 in cell quiescence and differentiation. Accumulation of p16 in senescent cells and its inhibitory role in regulating CDK4/CDK6/cyclinD1 complex formation suggests p16 could play a role in promoting G1 phase exit to senescence, quiescence, or differentiation. It might be suggested that p16, in addition to its proposed role in cell cycle senescence, may also play an important role in promoting quiescence and/or differentiation. We hypothesized that p16 plays a critical regulatory role that is required for cells to exit the cell proliferation cycle and for maintenance of these non-proliferative and post-proliferative states.

MATERIALS AND METHODS

CELL CULTURE

Parental canine mammary tumor cell lines (CMT12, CMT27, and CMT28) were originally obtained from Dr. L. Wolfe (30). Parental and single cell-derived clonal canine mammary tumor cell lines stably transfected with human p16 (CMT27A, CMT27H, CMT28A, and CMT28F) and primary NCF (normal canine fibroblast) cells were derived and cultured in L-15 medium (Gibco) with antibiotics (Sigma), and 10% FBS (Fetal bovine serum, Hyclone) in tissue culture flasks (Corning) at 37°C (95% air; 5% CO_2) as previously described [DeInnocentes et al., 2006]. Transfected CMT cell clones were cultured in L-15 medium (Gibco) with geniticin (100 μg/ml, Invitrogen) [DeInnocentes et al., 2009]. Cells were serum-starved by changing the FBS concentration from 10% to 0.5% in L-15 medium. For re-feeding, cultures were changed back to complete growth medium with 10% FBS.

PREPARATION OF RNA, PRIMER DESIGN, AND SEMI-QUANTITATIVE AND QUANTITATIVE RT-PCR

Cell cultures were grown to 75–80% confluence and total cellular RNA isolated using RNA Stat 60 (Tel-Test, Inc.). Concentration of RNA was determined by absorbance at 260 nm [You and Bird, 1995]. Canine p16, CDK4, CDK6, L37, cyclin D1, and p27 cDNA synthesis and amplification was performed by reverse transcriptase semi-quantitative and quantitative PCR using specific primers (Table I) [DeInnocentes et al., 2009]. Quantitative PCR was performed using a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System and assays were performed using an ABsoluteTM QPCR SYBR® Green Fluorescein Mix (Thermo Scientific). mRNA expression was analyzed by the comparative $\Delta\Delta C_t$ method. Amplicons were gel purified, cloned into vector pCR2.1 (Invitrogen) and identity was confirmed by sequencing (Auburn University Genomics and Sequencing Laboratory). RNA extraction, semi-quantitative analysis by RT-PCR, and sequencing was performed as previously described [DeInnocentes et al., 2006].

WESTERN BLOT

Cells were lysed with extraction buffer (1× PBS pH 7.0, 0.1% Tween-20, 0.1% NP-40, 100 mM PMSF). Three freeze/thaw cycles were performed to release proteins (8×10^6 cells) and lysates were cleared by centrifugation (14,000*g*) for 10 min at 4°C and

TABLE I. Primers Used for Reverse-Transcriptase PCR

Gene	Primer (5′–3′)	Genbank accession no.[a]	Amplicon size (bp)
L37 sense	AAGGGGACGTCATCGTTCGG	XM_844999	194
L37 anti-sense	AGGTGCCTCATTCGACCGGT		
p16 sense	AGCTGCTGCTGCTCCACGG	FJ542309	103
p16 anti-sense	ACCAGCGTGTCCAGGAAGCC		
Cyclin D1 sense	AGGAGCAGAAGTGCGAGGAG	AY620434	374
Cyclin D1 anti-sense	CACATCTGTGGCACAGAGCG		
CDK4 sense	AAGCCTCTCTTCTGTGGAAACTCT	XM_538252	223
CDK4 anti-sense	AGATTCGCTTGTGTGGGTTAAA		
CDK6 sense	AGGGCATGCCGCTCTCCACCATCC	XM_847267	353
CDK6 anti-sense	GATGCGGGCAAGGCCGAAGTCAGC		
p27 sense	CTGGAGCGGATGGACGCCA	AY004255	280
p27 anti-sense	TCTCCTGCGCCGGCACCT		

[a]Primers were designed based on canine cDNA sequences in Genbank.

supernatants stored at −80°C [Bird and Deinnocentes, 2004]. Supernatants were assayed for protein concentration using a BCA200 protein assay kit (Pierce). Protein extracted from each cell line was boiled in 1× Laemmli buffer (5× is 50% glycerol, 10% SDS, 2.56% β-mercaptoethanol, 2.13% 0.5 M Tris–HCl, pH 6.8, trace of bromophenol blue) for 10 min before loading onto a polyacrylamide electrophoresis gel (4–20% precise™ protein gels, Pierce) along with Kaleidoscope markers (range 10–250 kDa, BioRad). Electrophoresis was run in BupHTM Tris–HEPES–SDS running buffer (Pierce) for 1 h (100 V) and proteins were transferred to PVDF membranes (Immobilone®-P Transfer Membrane, Millipore) using a BupHTM Tris–Glycine Buffer Pack (Pierce) as transfer buffer for 2 h at 4°C and 300 mA. The membrane was washed in washing buffer (TBS-T Buffer, 20 mM Tris–HCl, 136 mM NaCl, 0.1% v/v Tween 20, pH 7.6). The membrane was blocked with blocking buffer (Pierce) for 2 h at room temperature. The membrane was incubated with multi-species, polyclonal anti-human rabbit p16 primary antibody (1:1,000; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Membranes were washed with washing buffer 4× for 10 min each. The membrane was incubated with anti-rabbit IgG-HRP secondary antibody (1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The membrane was washed again 4× for 10 min each. Western blot analysis was performed using the ECLTM Western blotting analysis system (Amersham Biosciences) according to the manufacturer's instructions. For GAPDH analysis, membranes were stripped by incubating with stripping buffer (Pierce) at room temperature. The membrane was then blocked as described above and incubated with mouse GAPDH primary antibody (1:5,000; AbD Serotec) for 4 h, and washed with wash buffer overnight. The membrane was incubated with anti-mouse IgG-HRP secondary antibody (1:10,000; Jackson Immunoresearch) for 1 h at room temperature. Western blot analysis was performed as described above.

^{3}H-THYMIDINE INCORPORATION ASSAY

Parental CMT cells, their p16 transfected clones, and NCF cells were grown as described above in 24-well cell culture plates (Corning) until 75% confluent. Medium was changed to 0.5% FBS serum starvation medium and 10 μCi/ml tritium-labeled thymidine (PerkinElmer) was added to individual wells at intervals noted in results. Cells were incubated for 4 h and washed with HBS (HEPES buffered saline; 10 mM HEPES pH 7.2, 0.9% NaCl) buffer twice. Cells were lysed with 200 μl TES (Tris–EDTA–SDS; 10 mM Tris–HCl pH 8.0, 2 mM EDTA, 1% SDS). Well contents (200 μl) were transferred to Whatman 540 paper discs and fixed [Bird et al., 1988]. Discs were washed in bulk in 300 ml for 20 min each in 20% (w/v) tricholoroacetic acid then in 10% (w/v) trichloroacetic acid, absolute ethanol, chloroform, and then absolute ethanol at room temperature. Discs were dried and immersed in scintillation liquid (ScintiSafe™ Econo 1, Fisher) and acid precipitable radioactivity determined in a liquid scintillation counter [Bird et al., 1988].

FLOW CYTOMETRY

CMT cells, their clones, and NCF cells were grown in 6-well plates (Corning) until 75% confluence. Medium was changed to 0.5% FBS medium at 75% confluence and the cells were harvested every 24 h after starvation with trypsin. Harvested cells were washed twice in HBS, and fixed with 70% ethanol [You and Bird, 1995]. Fixed cells were stained with propidium iodide, and cell cycle was analyzed by flow cytometry [DeInnocentes et al., 2006, 2009].

STATISTICAL ANALYSIS

All data comparisons between exponential and serum-starved cells were statistically analyzed using a two-sample Student's *t*-test.

RESULTS

mRNA EXPRESSION OF p16 AND ASSOCIATED GENES

Well characterized [DeInnocentes et al., 2009] parental canine mammary cancer cell lines (CMT28, CMT27, and CMT12) [Wolfe et al., 1986], clonal p16 transfected canine mammary tumor cell lines (CMT27A, CMT27H, CMT28A, and CMT28F) and normal canine fibroblasts (NCF) were used as models to investigate the role of p16 during quiescence [DeInnocentes et al., 2009; Bird et al., 2011]. Expression of mRNAs encoding p27, p16, CDK4, CDK6, cyclin D1, and L37 ribosomal protein genes were investigated using semi-quantitative RT-PCR. CMT27, CMT27A, CMT27H, CMT28, CMT28A, CMT28F, CMT12, and NCF cells all expressed mRNAs encoding p27, CDK4, CDK6, cyclinD1, and L37, while only NCF, CMT27A, CMT27H (weak/undetectable expression), CMT28, CMT28A, and CMT28F expressed p16 mRNA (Fig. 1A). This confirms our previous report that RT-PCR of CMT28 RNAs detected expression of p16 mRNA while RT-PCR of RNAs derived from CMT27 or CMT12 cell lines did not and also confirmed that p16 expression defects appear to be common in CMT cells. Surprisingly, only NCF and p16 transfected

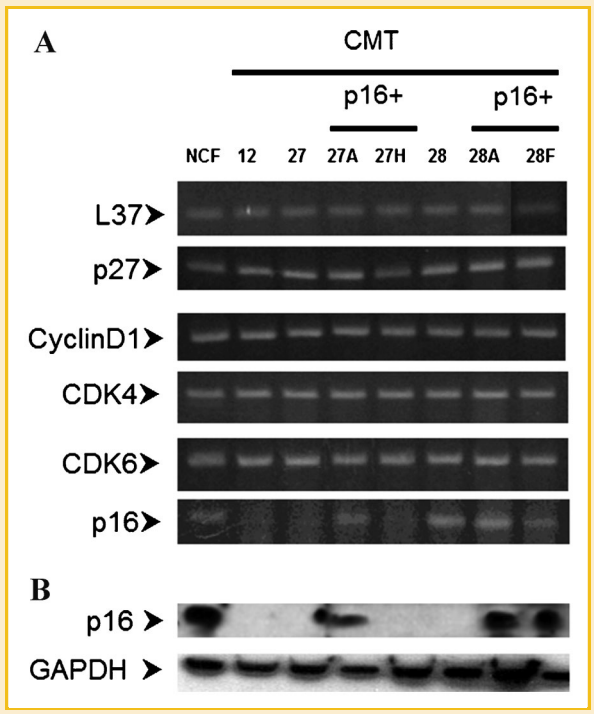

Fig. 1. Semi-quantitative reverse transcriptase PCR and Western blot of cell cycle proteins. A: mRNA levels of p16, CDK4, CDK6, cyclin D1, p27, and L37 (rtPCR/expression control) were evaluated in parental canine mammary tumor cell lines (CMT12, CMT27, CMT28), p16 transfected CMT cell clones (CMT27A, CMT27H, CMT28A, CMT28F), and normal canine fibroblasts (NCF). Transcripts were amplified from 1 μg RNA by RT-PCR and analyzed by agarose gel electrophoresis. B: p16 protein levels were analyzed by Western blot using polyclonal rabbit anti-human-p16 antibody (Santa Cruz Biotechnology, Inc.) and mouse anti-GAPDH antibody (AbD Serotec). Secondary antibodies used for p16 were anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Inc.) and for GAPDH were anti-mouse IgG-HRP (Jackson Immunoresearch).

cell lines CMT27A, CMT28A, and CMT28F translated p16 to detectable protein while CMT28 cells, although they express abundant p16 mRNA, did not synthesize detectable p16 protein (Fig. 1B). This suggests that CMT28 cells suffer a post-transcription defect blocking p16 translation and that all three parental CMT cell lines are p16 defective.

DNA REPLICATION AND CELL CYCLE EXIT FOLLOWING SERUM-STARVATION

To study the role of p16 expression in cell cycle exit and re-entry, we developed a cell model using serum starvation (to stop cell growth) and re-feeding (to initiate cell cycle re-entry) of CMT cell lines. The purpose was to deprive cells of required nutrients and growth factors provided by fetal bovine serum in culture medium to stop cell growth by inducing quiescence and then provide cells with growth nutrients to induce cell cycle re-entry with relative synchrony [Coller et al., 2006]. Cells were cultured to approximately 75% confluence and then serum-starved (0.5% FBS medium) until they stopped proliferating but before cells started floating in suspension. Cells were monitored for morphology and confluence daily. Cells were analyzed for cell cycle phase distribution by flow cytometry and DNA replication by ^{3}H-thymidine incorporation into acid-precipitable molecules. DNA replication in NCF, CMT12, CMT27, CMT27A, CMT27H, CMT28, CMT28A, and CMT28F decreased by 72 h post-serum starvation in comparison with proliferating cells (Fig. 2). Effects of serum-starvation were more profound in p16-transfected CMT27A cells in comparison to parental CMT27 cells where serum-starvation had a clear and significant inhibitory effect on DNA replication in p16 transfected CMT27A cells (Fig. 2).

Reduction in DNA replication after serum-starvation suggested some CMT cells had stopped dividing. To ensure that reduction in DNA replication and/or cell numbers was due to cell cycle exit to quiescence and not due to cell mortality, we analyzed the cell cycle phase distribution of cells by flow cytometry. CMT12, CMT28, CMT28A, CMT28F, CMT27A, CMT27H, and NCF cells exited cell cycle post-starvation and accumulated in G1 phase by 72 h in comparison to proliferating cells (Fig. 3). There was no enhanced cell accumulation in G1 phase in the case of CMT27 cells post-serum-starvation (Fig. 3B), although p16-transfected CMT27A and CMT27H cells re-acquired the ability to accumulate in G1 phase. CMT12, CMT27A, CMT27H, CMT28, CMT28A, CMT28F, and NCF cells all exhibited clear effects of serum-starvation suggesting transition out of the cell cycle while in comparison CMT27 demonstrated little effect of starvation (Fig. 3). This clearly implies that transfection of p16 rescued the ability of CMT27 cells to exit cell cycle post-starvation in CMT27A and CMT27H cells.

CELL CYCLE PHASE ANALYSIS FOLLOWING SERUM RE-FEEDING

To ensure that cells were entering the quiescent phase, and not irreversible senescence due to serum-starvation, all the arrested cell cultures were refed with serum containing growth medium. Growth media containing FBS was added to NCF, CMT12, CMT28, CMT28A, CMT28F, CMT27, CMT27A, and CMT27H cell cultures after 72 h of serum-starvation. Cells were harvested every 12 h following serum refeeding in all cell lines (Fig. 4). Cells accumulated in S and G2/M phases after 24–36 h of serum re-feeding of NCF, CMT27, CMT27A,

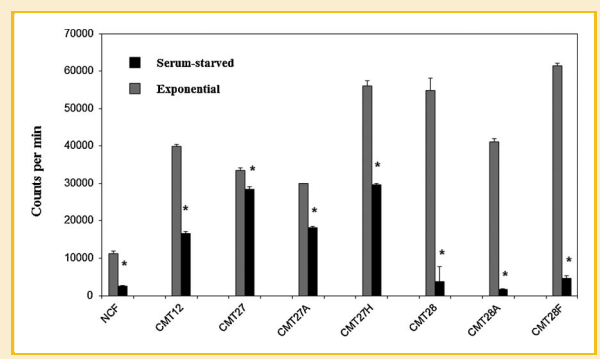

Fig. 2. ^{3}H-thymidine incorporation assay of DNA synthesis. Acid precipitable ^{3}H-thymidine incorporation in exponentially growing and 72 h serum-starved parental canine mammary tumor (CMT12, CMT27, and CMT28) cell lines, p16-transfected CMT clones (CMT27A, CMT27H, CMT28A, and CMT28F), and normal canine fibroblasts (NCF) were compared. Time of incubation for isotope incubation was 4 h. Asterisks indicate statistically significant differences comparing serum-starved and exponential cells as determined by two-sample Student's *t*-test ($^{*}P < 0.05$).

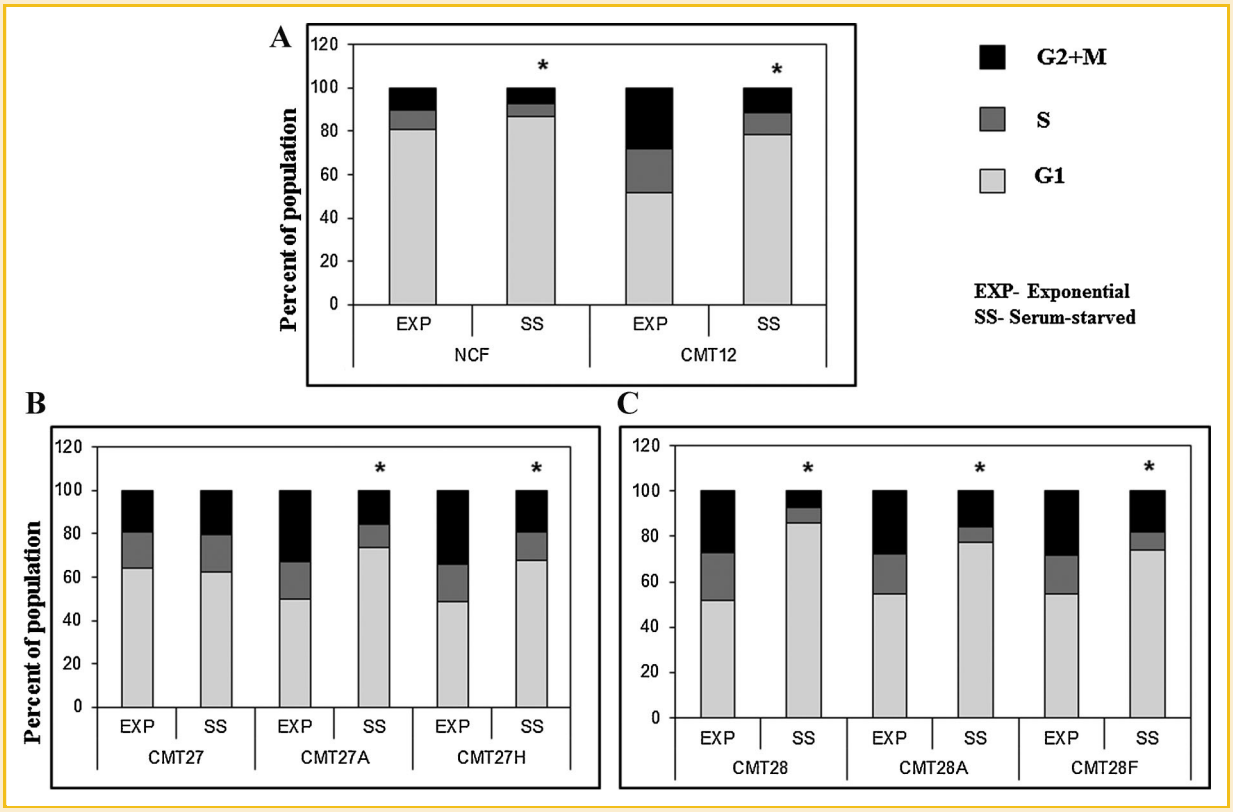

Fig. 3. Cell cycle phase analysis by flow cytometry following serum-starvation. Cells were harvested every 24 h post-serum-starvation and stained with propidium iodide and analyzed for cell cycle phase (G1, S, and G2/M phases) by flow cytometry on a linear fluorescence scale. A: Percent of population found in each cell cycle phase in NCF and CMT12, (B) in CMT27 and p16-transfected CMT27A and CMT27H cell clones, and (C) in CMT28 and p16-transfected CMT28A and CMT28F cell clones. Asterisks indicate statistically significant differences in cells present in G1 phase comparing serum-starved and exponential cell cultures as determined by the two-sample Student's *t*-test ($^*P < 0.05$).

CMT27H, CMT28, CMT28A, and CMT28F cells (Fig. 4). CMT12 cells did not alter their cell cycle phase distribution following serum re-feeding, which may suggest that CMT12 cells enter senescence post-serum starvation. In all the cell lines except CMT12 cells, an increase in relatively synchronous S phase re-entry was observed following serum refeeding of starved cells suggesting they had re-entered the cell cycle.

QUANTITATIVE RT-PCR ANALYSIS OF CKI EXPRESSION IN QUIESCENT CELL CULTURES

p27 expression has been reported to be upregulated during cellular quiescence [Sherr and Roberts, 1995] and therefore we have used p27 as a marker of cell quiescence. To further validate which cell lines were reaching quiescence, post-cell cycle exit due to serum-starvation, we compared p27 mRNA expression in exponentially proliferating and serum-starved cells using quantitative rtPCR and the comparative $\Delta\Delta C_t$ method. Ct values for p27 mRNA expression were compared between serum-starved and exponentially proliferating NCF, CMT12, CMT27, CMT27A, CMT27H, CMT28, CMT28A, and CMT28F cells and were normalized to expression of the L37 gene [Su and Bird, 1995]. By observing the normalized expression of p27, it was clear that only serum-starved NCF, CMT27A, and CMT28F overexpressed p27 significantly which demonstrated that only these cell lines likely achieved the cell quiescent cell phenotype (Fig. 5). Quantitative RT-PCR results showed that, along with p27, the p16 gene was also overexpressed significantly in the same quiescent NCF and CMT28F cells and marginally, but significantly, in quiescent CMT27A cells suggesting a possible role for p16 along with p27 in cell quiescence in these cell lines.

COMPARATIVE p16 PROTEIN EXPRESSION IN SERUM-STARVED AND EXPONENTIAL CELLS

To further evaluate the role of p16 in cell quiescence, p16 protein expression was compared between exponential and serum-starved cells by Western blot in NCF and CMT cell lines. Blots were scanned and normalized to expression of GAPDH protein. p16 protein was found to accumulate to higher normalized levels in serum-starved cells that expressed relative enhancements of p16 protein levels of 41% for NCF, 29% for CMT27A and 100% for CMT28F cells (Fig. 6). p16 protein accumulation in serum-starved quiescent cells also suggested that p16 may have a role in cell quiescence in normal and p16-transfected cancer cells.

DISCUSSION

The cyclin-dependent kinase inhibitors act as multifunctional cell proliferation inhibitors that integrate signals to suppress cell growth

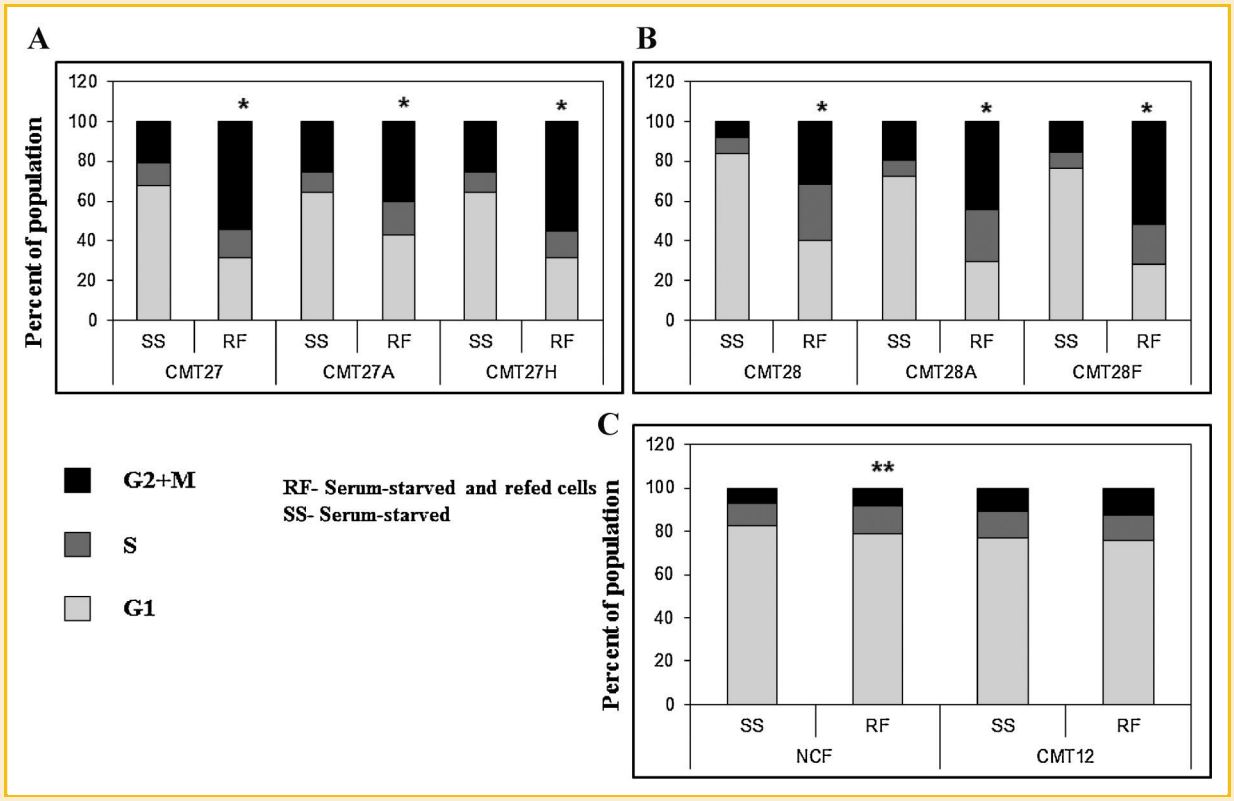

Fig. 4. Cell cycle phase analysis following serum-refeeding. Cells were harvested every 12 h post-serum-refeeding, stained with propidium iodide and analyzed for cell cycle phase by flow cytometry on a linear fluorescence scale. Percent of population found in each cell cycle phase (G1, S, and G2/M) in (A) parental CMT27 and p16-transfected CMT27A and CMT27H cell clones, (B) in parental CMT28 and p16-transfected CMT28A and CMT28F cell clones, and (C) in CMT12 and NCF cells. Asterisks indicate a statistically significant difference in cells present in G1 phase comparing serum-starved and exponential cells as determined by the two-sample Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.1$, respectively).

by inhibiting CDK function [Morgan, 1997]. The traditional role of p16 is inhibition of cell proliferation in G1 phase. Previous data suggests accumulation of p16 mRNA and protein occur as cells enter senescence [Zindy et al., 1997]. p21 acts as the mediator of cell cycle arrest prior to senescence while p16 appears to induce, maintain, and promote the senescent phenotype [Zindy et al., 1997; Stein et al., 1999]. Under the stimulus of stress and senescence, levels of p16 increase with decreased levels of histone-lysine *N*-methyltransferase EZH2 enzyme [Bracken et al., 2007]. Accumulation of p16 in senescent cells, and its inhibitory role in regulating CDK4/CDK6/cyclinD1 complex formation explains why expression of p16 could lead to arrest of cell cycle in G1 phase [Lea et al., 2003] but little is known regarding the role of p16 once cells enter quiescence.

Cell quiescence is a temporary undividing cell stage often occurring prior to terminal differentiation, which is characterized by lower metabolism and no DNA synthesis [Yusuf and Fruman, 2003]. Cell quiescence can be achieved experimentally by serum-starvation or contact-inhibition [Coller et al., 2006]. In the absence of growth factors, cells retire to G0 phase and are termed quiescent. Upon growth factor refeeding, cells re-enter the cell cycle. We have used this phenomenon to observe the effects of p16 on entry and exit from cell quiescence.

In this investigation we have used well-characterized CMT cell lines [Wolfe et al., 1986] and normal canine fibroblasts (NCF) to model cell cycle exit and re-entry [DeInnocentes et al., 2006; Bird et al., 2009]. The CMT cell lines used lack p16 expression either at the mRNA or protein expression levels, while p27 and other proteins associated with regulation of cell cycle progression such as; CDK4, CDK6, cyclin D1, and Rb are expressed at near normal levels in all of the cell lines. In addition to parental CMT cell lines, we also employed derived p16-transfected cloned cell lines [DeInnocentes et al., 2009]. Many of these clones exhibited phenotype rescue of the neoplastic phenotype to varying degrees due to p16 transfection making these mammary cancer cell lines particularly interesting for evaluation following serum-starvation [DeInnocentes et al., 2009].

Decreased levels of cyclin D1, increased levels of p27, and accumulation of unphosphorylated Rb are the hallmarks of cell quiescence [Sherr and Roberts, 1995; Boonstra, 2003; Pajalunga et al., 2007] and CKIs p27 and p21 have been reported to play important roles in cell quiescence. p27 induces and maintains cell quiescence by sequestering the CDK/cyclin D1 complex [Carroll et al., 2003; Georgia and Bhushan, 2006]. p21 has been reported to be important for cell quiescence in development of mouse hematopoietic stem cells and quiescence in human foreskin fibroblasts is suppressed in its absence [Cheng et al., 2000; Pajalunga et al., 2007]. p16 has also been reported to induce a G0-like state in hematopoietic cells [Furukawa et al., 2000]. We have observed that p16 expressing cell lines (both mRNA and protein), such as NCF, CMT28A, CMT28F, and CMT27A, do exit cell cycle

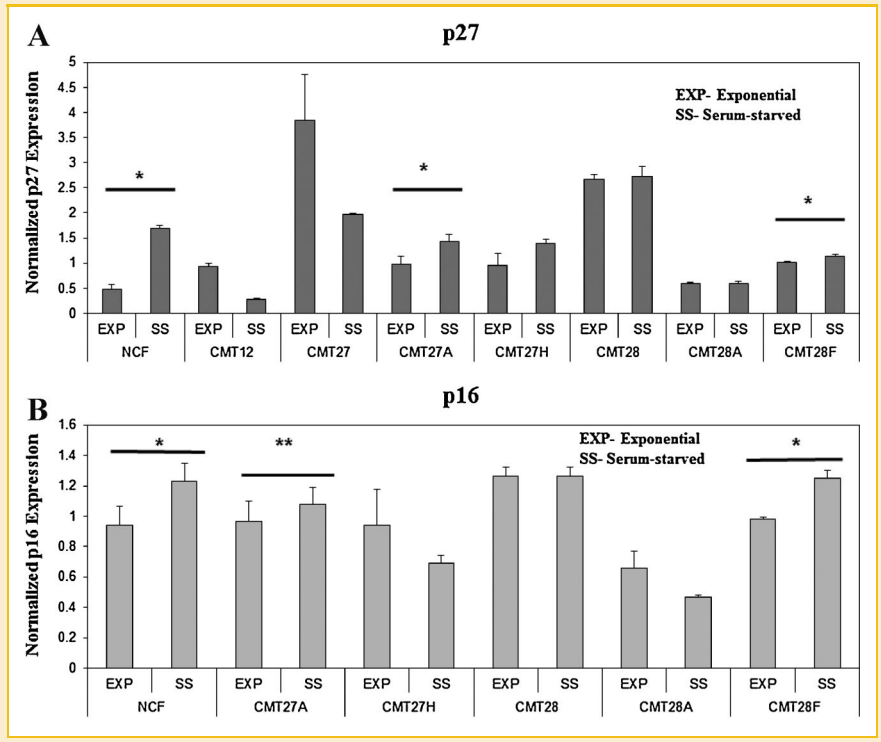

Fig. 5. Quantitative RT-PCR analysis of p27 and p16 expression in exponential and serum-starved cells. A: Analysis of p27 mRNA transcript; and B: p16 mRNA transcript levels. Asterisks indicate statistically significant differences between serum-starved and exponential cells as determined by the two-sample Student's *t*-test ($^{*}P < 0.05$, and $^{**}P < 0.1$, respectively). Expression levels were normalized to levels of control L37 expression.

post-serum-starvation while CMT27 cells, which lack p16 expression, do not exit cell cycle post-serum starvation. The continued growth of CMT27 cells after removal of growth factors from the media suggested that failure to exit cell cycle was due to some other defect, as these cells did not appear to arrest and become quiescent in the absence of FBS. CMT12 cells, also lacking p16 expression, demonstrated cell cycle exit but did not show synchronous cell cycle re-entry. This suggests that CMT12 cells may exit cell cycle permanently and may reach senescence post-serum starvation. However, CMT28 cells, which lack p16 expression can exit cell cycle post-serum starvation but do not appear to reach quiescence as there was no upregulation of p27 mRNA observed in these cells. This suggests that factors other than p16, such as p27 possibly, may be responsible for cell cycle exit in CMT28 cells. From these observations, we conclude that most cell lines expressing p16 protein do react to serum-starvation by exiting cell cycle although an alternative pathway likely exists as p16 negative cells, such as parental CMT28 cells, still appear to arrest and then re-enter the cell cycle following serum refeeding.

CMT28 cells have also been reported to overexpress c-*erb*B-2 in comparison to CMT12 and CMT27 [Ahern et al., 1996]. We have speculated that transformation in CMT28 cells is promoted by upregulated expression of the c-*erb*B-2 receptor pathway making them relatively independent of growth factor stimulation. Parental CMT28 cells and p16-transfected CMT28A and CMT28F cell clones were all capable of exiting the cell cycle in response to serum-starvation even though parental CMT28 cells do not express p16. Lack of p16 expression has been associated with cancer [Gruis et al., 1995; Swellam et al., 2004], while p16 overexpression has been reported in cell senescence [Agherbi et al., 2009] and cell cycle exit [Kivinen et al., 1999; Wang et al., 2008]. It has also been reported

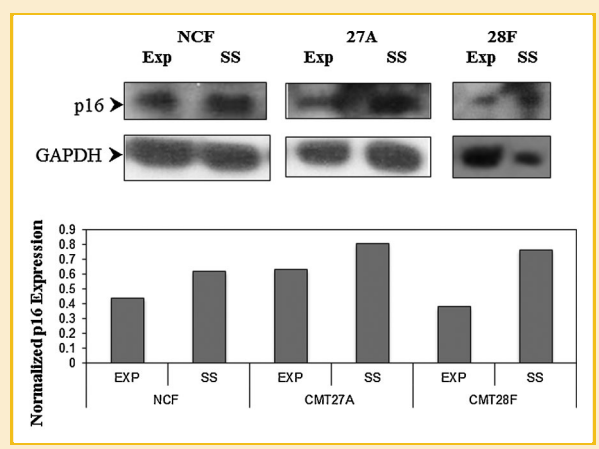

Fig. 6. Western blot analysis of p16 and GAPDH protein expression in exponential and serum-starved NCF, CMT27A, and CMT28F cells. Primary antibodies used for p16 were rabbit anti-p16 (Santa Cruz Biotechnology, Inc.) and for GAPDH were mouse anti-GAPDH (AbD Serotec). Secondary antibodies used for p16 were anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Inc.) and for GAPDH were anti-mouse IgG-HRP (Jackson Immunoresearch). Western blots were scanned, quantified and data plotted as normalized to GAPDH protein expression for the same sample. A representative experiment is shown.

that ectopic expression of p16 prevents re-entry into the cell cycle [Lea et al., 2003]. Our results with p16-transfected CMT28 cells demonstrate that this is likely dependent on context as these cells could re-enter the cell cycle. The effects of ectopic expression of p16 were also evident in our model of cell cycle exit and re-entry. p16-transfected CMT27A cells reacquired the capability of exiting cell cycle post-serum starvation in contrast to parental CMT27 cells. Rescue of cell cycle exit in p16-transfected cells strongly suggests an important role for p16 in cell cycle exit comparable to the effect observed in normal canine fibroblasts post-serum starvation. Because all of the p16 expressing cells and clones (NCF, CMT27A, CMT28A, and CMT28F) re-entered the cell cycle post-serum refeeding, these cells must have entered a transient undividing phase post-serum-starvation: most likely quiescence.

Suppression of proliferation is thought to be dependent on upregulated levels of p16 and p27, which render such cells unable to activate cyclin D1, cyclin E and their associated cyclin-dependent kinases [Izzard et al., 2002]. Upregulated levels of p27 mRNA have also been reported in cell quiescence making it an informative marker of cell quiescence. While each of the NCF, CMT27A, CMT27H, CMT28, CMT28A, and CMT28F cells demonstrated cell cycle exit post-serum starvation, only NCF, CMT27A, and CMT28F expressed upregulated levels of p27 mRNA and thus could be characterized as quiescent. This suggested that only these cell lines achieved authentic quiescence while the other cell lines (CMT27H, CMT28, and CMT28A cells) may have only progressed to a non-proliferative pre-quiescent stage. Quiescent NCF, CMT27A, and CMT28F cells expressed upregulated levels of p16 mRNA post-serum starvation coincidentally with elevated p27 mRNA expression. This suggested that p16 might also play a role in maintaining cell quiescence following cell cycle exit. p16 protein expression was elevated in all p16 expressing cell lines including NCF, CMT27A, CMT28A, and CMT28F cells. CMT28A cells did not express upregulated levels of p16 mRNA but did express elevated levels of p16 protein post-serum starvation. This further suggests p16 plays a role in cell cycle exit and that an important p16 partner has yet to be identified in quiescent cells. In contrast, serum starvation induced p27 expression was not evident in CMT27H clones. The difference between the two sets of clones may be attributed to absence of p16 mRNA and protein in CMT27H cells.

Cell cycle exit, due to serum-starvation and subsequent accumulation of p16 mRNA and protein demonstrated the effects of p16 on cell quiescence and its role in cell cycle exit. Until, now, p16 has been primarily associated with cell senescence and cell cycle exit and not with cell quiescence. We have shown that p16 also has a potential role in cell quiescence because, along with cell cycle exit, ectopic expression of p16 can also promote cells to enter a quiescent phenotype.

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