

Lactoferrin Down-Regulates G₁ Cyclin-Dependent Kinases during Growth Arrest of Head and Neck Cancer Cells

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ABSTRACT

The molecular mechanism of lactoferrin-induced cell growth inhibition is incompletely understood. Studying head and neck cancer cells treated with human lactoferrin, we observed growth arrest in three of four cell lines tested. This growth arrest was caused by cell cycle inhibition at the G₀-G₁ checkpoint. Lactoferrin-induced growth inhibition was associated with a large increase in p27 protein, accompanied by decreased phosphorylation of retinoblastoma protein, and suppression of cyclin E. Decreased levels of phosphorylated Akt were also observed in lactoferrin-sensitive cell lines after treatment. These findings suggest that in head and neck cancer cells the growth inhibitory effects of lactoferrin are mediated through a p27/cyclin E-dependent pathway that may be modulated in part by changes in Akt phosphorylation.

INTRODUCTION

Lactoferrin is an iron binding glycoprotein that is mainly found in external secretions such as breast milk. Lactoferrin is a component of the nonspecific immune system, having antimicrobial properties against bacteria, fungi, and several viruses (1). Recently, some studies have suggested chemopreventive and antitumor activity for lactoferrin as well. In rat models, lactoferrin treatment significantly reduced azoxymethane-induced colonic carcinogenesis, as well as *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced bladder carcinogenesis (2, 3). In addition, Bezault *et al.* (4) found that lactoferrin reduced solid tumor growth and strongly inhibited experimental metastasis in mice. Elimination of natural killer cell activity by pretreatment of mice with anti-asialo GM1 antibody abrogated these effects, suggesting that the antitumor activity of lactoferrin might be mediated through natural killer cells. More recent studies suggest that oral lactoferrin may act as an immunomodulatory agent by stimulating production of interleukin 18 and other cytokines by gut enterocytes (5). Lactoferrin may also inhibit tumor-

mediated angiogenesis. Lactoferrin exerts an antiproliferative effect on endothelial cells *in vitro*, and significantly inhibits the vascular endothelial growth factor-mediated angiogenic response *in vivo* (6). In addition, Campbell *et al.* (7) have shown that lactoferrin may be down-regulated in some cancers, such as human breast carcinoma, and suggested that lactoferrin may regulate cell proliferation. This hypothesis was additionally supported by Damiens *et al.* (8), who demonstrated that treatment of human breast cancer cells with lactoferrin resulted in growth arrest at the G₁ to S transition of the cell cycle and induced an increase in cyclin-dependent kinase (CDK) inhibitor p21^{cip1} protein levels by a p53-independent mechanism. Thus, lactoferrin's antitumor effects may be mediated by multiple mechanisms.

In this study, we focused our attention on lactoferrin's effects on cell cycle progression in head and neck cancer cell lines. We found that treatment of 011, 012, and 022 head and neck cancer cells with lactoferrin-induced growth arrest at the G₁ to S transition of the cell cycle, whereas 019 head and neck cancer cells, which contain p53 and PTEN mutations, did not experience growth arrest. To additionally understand the molecular mechanism underlying this inhibition, we examined the effect of lactoferrin on the protein levels of key genes involved in the G₁ to S transition. Our data suggest that the growth inhibitory effects of lactoferrin are mediated through a p27/cyclin E-dependent pathway that may be modulated by changes in Akt phosphorylation.

MATERIALS AND METHODS

Reagent. Recombinant human lactoferrin was obtained from Agennix, Inc., and stored in solution (100 mg/mL) at -20°C.

Cell Lines. Four head and neck cancer cell lines (011, 012, 019, and 022) were previously established at the Johns Hopkins Hospital from head and neck cancers treated definitively by surgical resection and deposited at the American Type Culture Collection (Manassas, VA). These cell lines were maintained in a humidified atmosphere of 5% CO₂ in air at 37°C. The cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) and 100 µg/mL penicillin/streptomycin.

Flow Cytometry for Cell Cycle Analysis. Cells grown to ~70% confluence in 25-cm² flasks were treated with 10 µmol/L lactoferrin for 8, 17, 25, 32, 40, 48, 56, or 65 hours. Adherent cells were trypsinized and fixed in 70% ethanol at 4°C. Before analysis, cells were washed once in PBS and resuspended in 1 mL of propidium iodide working solution (PBS, 0.5 mg/mL RNase, and 0.1 mg/mL propidium iodide). Cell cycle distribution was determined by flow cytometry using a FACScan cytofluorimeter and the CellQuest Software program (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Received 5/19/04; revised 9/3/04; accepted 9/10/04.

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Table 1 Flow cytometry analysis of untreated (control) and 10 $\mu\text{mol/L}$ lactoferrin-treated 011, 012, 019, and 022 cells

Cell line	Treatment	Phase of cell cycle *		
		%G ₀ -G ₁	%S	%G ₂ -M
011	Control/25 h	59	30	11
	10 $\mu\text{mol/L}$ lactoferrin/25 h	72	18	10
012	Control/25 h	45	45	10
	10 $\mu\text{mol/L}$ lactoferrin/25 h	73	16	10
019	Control/42 h	42	27	31
	10 $\mu\text{mol/L}$ lactoferrin/42 h	46	29	25
022	Control/25 h	48	30	22
	10 $\mu\text{mol/L}$ lactoferrin/25 h	63	16	21

* Numbers indicate the percentage of cells in the different phases of the cell cycle.

Western Blot Analysis. Total cell lysates were prepared as described previously (9). Protein concentrations were determined with the Bio-Rad protein assay reagent with bovine serum albumin as a standard. Twenty to 30 μg samples of total cell lysates were fractionated by 4 to 20% SDS/PAGE and transferred to nitrocellulose membranes. After incubating for 1 hour in blocking buffer containing 5% nonfat dry milk in 1 \times PBS and 0.1% Tween 20 (1 \times Tween-Tris-buffered saline for pAkt), blots were probed for 2 hours at room temperature (overnight at 4°C for pAkt) with primary antibodies against cyclin E, CDK4, the CDK inhibitors p19, p21, p27, and p57, and transforming growth factor (TGF)- β (BD Biosciences, San Diego, CA), p53 (NeoMarkers, Inc., Fremont, CA), phosphorylated retinoblastoma protein (Rb), Akt, phosphorylated Akt (Cell Signaling Technology, Beverly, MA), and PTEN (Cascade Bio-Sciences, Winchester, MA). Blots were then washed and incubated with secondary antibody (donkey antirabbit or sheep antimouse immunoglobulin linked to horseradish peroxidase, Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hour at room temperature. After washing with Tween-PBS, bound antibody was visualized by fluorography with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). The amount of bound antibody was quantitated by densitometric analysis of scanned images by NIH Image.¹

RESULTS

Analysis of Cell Cycle Distribution. Four head and neck cancer cell lines were treated with lactoferrin for various times ranging from 8 to 42 hours. Compared with untreated cells, treatment with lactoferrin (10 $\mu\text{mol/L}$) for 25 hours induced growth arrest at the G₀-G₁ phase of the cell cycle in 011 (from 59 to 72%), 012 (from 45 to 73%), and 022 cells (from 48 to 63%), with a comparative drop in the S-phase from 30 to 18%, 45 to 16%, and 30 to 16%, respectively. However, lactoferrin had no effect on 019 cells even after 42 hours of treatment (Table 1). Testing increased amounts of lactoferrin (100 and 200 $\mu\text{mol/L}$) in all four head and neck cancer cell lines, we found no additional effect on growth arrest or the timing of this arrest at the G₁ to S transition of the cell cycle (data not shown). Comparable effects were also observed

when cells were presynchronized by serum starvation and then treated with lactoferrin after release, confirming the antiproliferative activity of lactoferrin (data not shown).

Effect of Lactoferrin Inhibition on Protein Levels Regulating the G₁ to S Transition of the Cell Cycle. To address the question of how lactoferrin-induced cell cycle arrest, we tested the expression of a number of molecules that regulate the transition from G₀-G₁ to S phase in two lactoferrin-sensitive cell lines (012 and 022) and one resistant cell line (019). Molecules tested included cyclin E, CKD4, various CDK inhibitors, and phosphorylated Rb.

Compared with control cells, lactoferrin treatment (10 $\mu\text{mol/L}$) resulted in a strong reduction in the expression of cyclin E in both 012 and 022 cells (Fig. 1, A and C) but had no effect on cyclin E levels in 019 cells (Fig. 1B). A significant decrease in the phosphorylation of the Rb protein was also clearly seen after 24-hour lactoferrin treatment in the 012 and 022 cells but not in 019 cells (Fig. 1A-C). These data are consistent with the flow cytometry data showing no arrest in the latter cell line.

Given these results, we next searched for changes in CDK inhibitor protein levels. We found that after lactoferrin treatment there was a 7-fold increase in p27 protein levels in 022 cells (Fig. 1C) and an almost 5-fold increase in 012 cells (Fig. 1A). On the other hand, p27 expression decreased between 10 and 65% in both treated and untreated 019 cells at all treatment time points as compared with the start of the experiment (Fig. 1B), in keeping with the absence of growth arrest observed in these cells. Slight increases in p21 and p57 protein levels were also observed in both 012 and 022 cells but not in the resistant 019 cells. Conversely, lactoferrin treatment decreased levels of the INK4 CDK inhibitor p19 by ~50% in 012 and 022 cells (Fig. 1A-C). CDK4 protein levels were unchanged in all cell lines after treatment (data not shown).

Investigating p53 protein expression we found that 019 cells expressed a mutant p53 allele, but we did not observe any change in p53 abundance in any of the cell lines tested after lactoferrin treatment (Fig. 2) to account for the observed changes in CDK inhibitors. Because p27 abundance can be affected by changes in mitogenic growth factors, as well as the phosphatidylinositol 3'-kinase/Akt pathway, we also examined TGF- β , PTEN, Akt, and phosphorylated Akt protein levels. After 24 hours of lactoferrin treatment, when growth arrest was observed, we found no consistent change in TGF- β in 012 and 022 cells. Total Akt protein levels in 012, 019, or 022 cells were unchanged as was PTEN expression in 012 and 022 cells. However, PTEN expression was absent in 019 cells. A significant decrease in pAkt was observed in the 012 and 022 cell lines (~2- and 6-fold, respectively), both of which experienced G₀-G₁ growth arrest (Fig. 3).

DISCUSSION

Chemoprevention and chemotherapy with naturally occurring compounds such as lactoferrin have become increasingly important strategies in inhibiting carcinogenesis and tumor growth. To better understand one possible mechanism of lactoferrin-mediated antitumor activity in head and neck cancer cells, we investigated how the cell cycle is affected by lactoferrin treatment. Treatment with lactoferrin resulted in a significant growth arrest in the G₀-G₁ phase of the cell cycle in three of four

¹ Internet address: <http://rsb.info.nih.gov/nih.image/>.

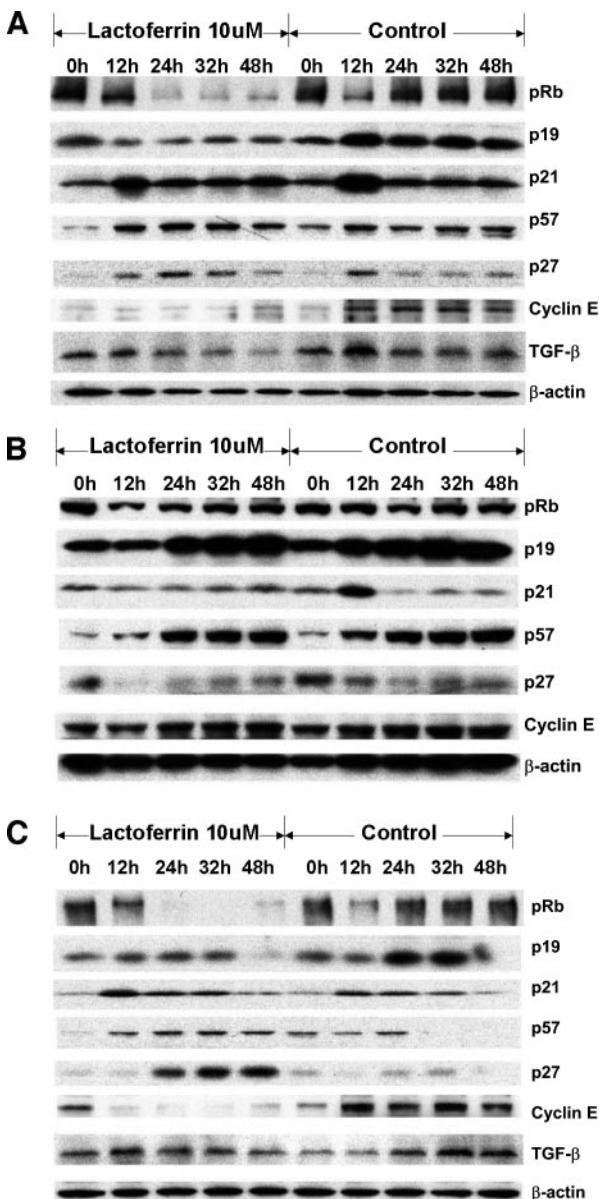


Fig. 1 Protein levels of cyclin E, pRb, the CDK inhibitors p19, p21, p27, p57, and β -actin in untreated (control) and 10 μ mol/L lactoferrin-treated O12 cells (A), O19 cells (B), and O22 cells (C). Protein levels of TGF- β are also shown in O12 and O22 cells. Treated cells were exposed to lactoferrin for different time intervals (0, 7 or 12, 24, 32, 48 hours). Protein levels were detected by Western blot analysis.

head and neck cancer cell lines studied. This arrest was associated with a decrease in the S phase of these cells. Given lactoferrin's low bioavailability (5), we expect that the concentration of lactoferrin required in our studies to induce growth arrest would not be achieved after oral administration of the protein. In addition, this concentration is somewhat higher than that used by Damiani *et al.* (8) to induce G₁ arrest in breast cancer cells. However, this concentration is within the range of concentrations reached in urine after oral administration (3) or in serum after i.v. dosing (10).

To additionally examine the molecular mechanisms underlying lactoferrin-induced cell cycle arrest, we tested the levels of key regulatory proteins required for transition past the G₁ restriction point of the cell cycle. Accumulating evidence suggests that cyclins and their antagonists, the CDK inhibitors, play a key role in the induction and inhibition of cell cycle progression (11–16). In mammalian cells, the active complexes in G₁ contain cyclin D–CDK4/6, cyclin A, and cyclin E–CDK2 (12). Cyclin D functions upstream of the Rb protein by binding to CDK4 or CDK6 leading to Rb phosphorylation. The phosphorylation of Rb in mid-to-late G₁ releases the transcription factors bound by Rb, resulting in their subsequent binding to the promoter regions of various genes leading to DNA synthesis (17). This is followed by assembly and activation of cyclin E and CDK2. Cyclin A and CDK2 then modulate additional steps required for DNA synthesis in S phase (18–21). CDKs also interact with a family of CDK inhibitors. To date, CDK inhibitors have been classified into two groups, based on similarities in sequence and action. One group, the CIP/KIP family, includes the inhibitors p21, p27, and p57, which appear to bind and inhibit the kinase activities of cyclin D/CDK4, cyclin D/CDK6, cyclin E/CDK2, and cyclin A/CDK2 (22–24). A second group, the INK4 family, includes p15, p16, p18, and p19 (25). These CDK inhibitors specifically bind CDK4 and CDK6 and prevent the association of the CDKs with their cyclin partners.

In O12 and O22 cells, treatment with lactoferrin for 24 hours resulted in large increases in p27 protein, along with substantially smaller increases in p21 and p57. This small increase in p21 contrasts with the findings of Damiani *et al.* (8), who attributed lactoferrin-induced growth arrest in human breast cancer cells to a

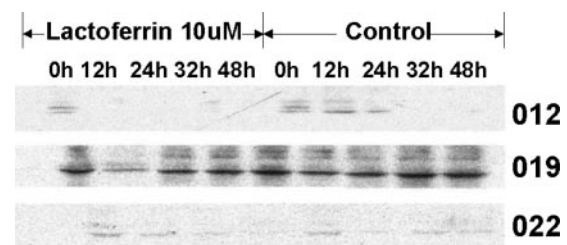


Fig. 2 Protein levels of p53 in untreated (control) or 10 μ mol/L lactoferrin-treated O12, O19, and O22 cells. Treated cells were exposed to lactoferrin for different time intervals (0, 7 or 12, 24, 32, 48 hours). Protein levels were detected by Western blot analysis.

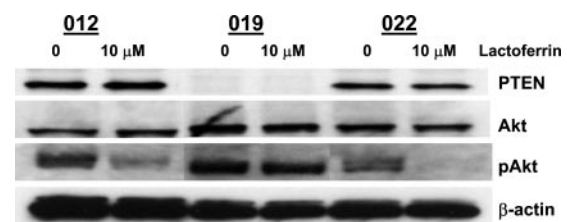


Fig. 3 Protein levels of Akt, PTEN, pAkt, and β -actin in untreated (control) or 10 μ mol/L lactoferrin-treated O12, O19, and O22 cells as detected by Western blot analysis. Treated cells were exposed to lactoferrin for 24 hours.

p53-independent increase in p21 protein. Although the observed increases in all three CIP/KIP family members, p21, p57, and p27, may have contributed in some part to the decreased phosphorylation of Rb and reduction in cyclin E, given that the largest change was seen in p27 protein, we focused further attention there.

Although transcriptional regulation of p27 is seen, p27 regulation occurs predominantly at the levels of translation and protein stability (26). This is consistent with our observation that p27 mRNA levels were unchanged in treated compared with control cell lines (data not shown), suggesting that lactoferrin treatment blocked p27 degradation by inhibiting proteolysis. p27 protein levels can be altered by changes in a number of pathways, including the mitogenic TGF- β pathway and the phosphatidylinositol 3'-kinase/Akt pathway. TGF- β down-regulates the steady-state levels of p27 protein (27). Decreases in phosphorylated Akt cause decreased p27 phosphorylation and increased resistance of the protein to proteasomal degradation (28). Increased p27 protein levels were not associated with a change in TGF- β protein in either 012 or 022 cells. However, we did see a substantial decrease in pAkt in 012 and 022 cells. Furthermore, lactoferrin treatment did not induce cell cycle arrest nor alter p27 protein levels in the PTEN(-/-) 019 cell line in which Akt would be expected to remain constitutively active (phosphorylated; ref. 29). Our findings, therefore, are most consistent with a decrease in pAkt inducing an increase in p27 protein in our lactoferrin-sensitive cells.

In conclusion, in select head and neck cancer cell lines, lactoferrin induces G₀-G₁ cell cycle arrest that is likely mediated by a decrease in pAkt, causing a marked increase in p27 and a reduction in cyclin E and pRb protein levels. Lactoferrin has been shown to bind to insulin-like growth factor-binding protein-3 (30). Hence, it may act by altering the availability or stability of insulin-like growth factor, a known regulator of the phosphatidylinositol 3'-kinase/Akt pathway (31). Delineation of the role of these, as well as additional potential upstream mediators, will require additional exploration.

REFERENCES

- van der Strate BWA, Beljaars L, Molema G, Harmsen MC, Meijer DKF. Antiviral activities of lactoferrin. *Antiviral Res* 2001;52:225-39.
- Sekine K, Watanabe E, Nakamura J, et al. Inhibition of azoxymethane-initiated colon tumor by bovine lactoferrin administration in F344 rats. *Jpn J Cancer Res* 1997;88:523-6.
- Masuda C, Wanibuchi H, Sekine K, et al. Chemopreventive effects of bovine lactoferrin on N-butyl-N-(4-hydroxybutyl)nitrosamine-induced rat bladder carcinogenesis. *Jpn J Cancer Res* 2000;91:582-8.
- Bezault J, Bhimani R, Wiprovnick J, Furmanski P. Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. *Cancer Res* 1994;54:2310-12.
- Varadhachary A, Wolf JS, Petrak K, et al. Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy. *Int J Cancer* 2004;111:398-403.
- Norryby K, Mattsby-Baltzer I, Innocenti M, Tuneberg S. Orally administered bovine lactoferrin systemically inhibits VEGF(165)-mediated angiogenesis in the rat. *Int J Cancer* 2001;91:236-40.
- Campbell T, Skilton RA, Coombes RC, Shousha S, Graham MD, Luqmani YA. Isolation of a lactoferrin cDNA clone and its expression in human breast cancer. *Br J Cancer* 1992;65:19-26.
- Damiens E, Yazidi IE, Mazurier J, Duthille I, Spik G, Boilly-Marer Y. Lactoferrin inhibits G₁ cyclin-dependent kinases during growth arrest of human breast carcinoma cells. *J Cell Biochem* 1999;74:486-98.
- Hibi K, Trink B, Patturajan M, et al. AIS is an oncogene amplified in squamous cell carcinoma. *Proc Natl Acad Sci USA* 2000;97:5462-7.
- Zimecki M, Artym J, Chodaczek G, Kocieba M, Kruzel ML. Protective effects of lactoferrin in Escherichia coli-induced bacteremia in mice: relationship to reduced serum TNF alpha level and increased turnover of neutrophils. *Inflamm Res* 2004;53:292-6.
- Pines J. Cyclins and cyclin-dependent kinases: a biochemical view. *Biochem J* 1995;308:697-711.
- Sherr CJ. G₁ phase progression: cycling on cue. *Cell* 1994;79:551-5.
- Grana X, Reddy EP. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* 1995;11:211-9.
- Sherr CJ, Roberts JM. Inhibitors of mammalian G₁ cyclin-dependent kinases. *Genes Dev* 1995;9:1149-63.
- Prietzsch H, Brock J, Kleine HD, Liebe S, Jaster R. Interferon-alpha inhibits cell cycle progression by Ba/F3 cells through the antagonism of interleukin-3 effects on key regulators of G₁-S transition. *Cell Signal* 2002;14:751-9.
- Olashaw N, Pledger WJ. Paradigms of growth control: relationship to Cdk activation. *Sci STKE* 2002;2002:1-14.
- Harbour JW, Dean DC. Rb function in cell-cycle regulation and apoptosis. *Nat Cell Biol* 2000;2:65-7.
- Koff A, Giordano A, Desai D. Formation and activation of a cyclin E-cdk2 complex during the G₁ phase of the human cell cycle. *Science (Wash. DC)* 1992;257:1689-94.
- Dulic V, Lees E, Reed SI. Association of human cyclin E with a periodic G₁-S phase protein kinase. *Science (Wash. DC)* 1992;257:1958-61.
- Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI, Weinberg RA. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* 1992;70:993-1006.
- Hatakeyama M, Brill JA, Fink GR, Weinberg RA. Collaboration of G₁ cyclins in the functional inactivation of the retinoblastoma protein. *Genes Dev* 1994;8:1759-71.
- Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G₁ cyclin-dependent kinases. *Cell* 1993;75:805-16.
- Polyak K, Lee MH, Erdjument-Bromage H, et al. Cloning of P27kip1, a cyclin-cdk inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 1994;78:59-66.
- Lee MH, Reynisdottir I, Massague J. Cloning of p57kip2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* 1995;9:639-49.
- Serrano M, Hannon GJ, Beach DA. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature (Lond.)* 1993;366:704-7.
- Alkarain A, Slingerland J. Deregulation of p27 by oncogenic signaling and its prognostic significance in breast cancer. *Breast Cancer Res* 2004;6:13-21.
- Ravitz MJ, Yan S, Herr KD, Wenner CE. Transforming growth factor beta-induced activation of cyclin E-cdk2 kinase and down-regulation of p27Kip1 in C3H 10T1/2 mouse fibroblasts. *Cancer Res* 1995;55:1413-6.
- Liu W, Asa SL, Fantus IG, Walfish PG, Ezzat S. Vitamin D arrests thyroid carcinoma cell growth and induces p27 dephosphorylation and accumulation through PTEN/akt-dependent and -independent pathways. *Am J Pathol* 2002;260:511-9.
- Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci USA* 1999;96:4240-5.
- Baumruher CR, Gibson CA, Schanbacher FL. Bovine lactoferrin binds to insulin-like growth factor-binding protein 3. *Domest Anim Endocrinol* 2003;24:287-303.
- Maier B, Gluba W, Bernier B, et al. Modulation of mammalian lifespan by the short isoform of p53. *Genes Dev* 2004;18:306-19.